REMARKS

Status of the Claims

Claims pending in the above-identified application are Claims 1, 3-5, and 17-24. Claims 1, 3-5, and 22-24 are amended. The amended claims do not introduce new matter into the above-identified application. Support for the amended claims is found throughout the specification.

Information Disclosure Statement

Applicant encloses herewith complete copies of References 14 and 16 cited in the Information Disclosure Statement filed on April 26, 2002.

The Rejection Under 35 USC § 112

Claims 1, 3-5, and 17-24 are rejected under 35 U.S.C. § 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which Applicant regards as his invention. Specifically, the Examiner rejects Claims 1 and 22-24 for failing to set forth steps for determining whether the compound affects cell proliferation. Further, Claims 1 and 22-24 are rejected due to the recitation of "compounds" as opposed to "a compound." Finally, Claims 5 and 17-19 are rejected as indefinite for reciting "a compound," which is inconsistent with the preamble of Claim 1. Applicant respectfully submits that the above amendments obviate the rejection of Claims 1, 3-5, and 17-24 under 35 U.S.C. § 112, second paragraph and requests that these rejections be withdrawn.

Claims 1, 3-5, and 17-24 are rejected under 35 U.S.C. § 112, first paragraph, as containing subject matter which was not described in the specification at the time the application was filed. Specifically, the Examiner rejects Claims 1 and 22-24 due to the inclusion of the phrase "having unknown cellular proliferative activity." Applicant respectfully traverses this rejection.

The specification states on page 7, lines 20-24, that "[i]n the assays of the present invention, the compound initially has unknown activity, effect or effects. The activity of the compound is unknown, in that the compound's effects in the assays of the present invention are not yet determined. The compound may have many other known activities, and may be a compound that has other therapeutic uses" (emphasis added). Based upon the PTO's position, it appears that the PTO is mandating exact or *in haec verba* disclosure of the phrase "having unknown cellular proliferative activity." Such a mandate is not required by the Court of Appeals for the Federal Circuit and the PTO through its Guidelines for Examination of Patent Applications under 35 U.S.C. § 112, first paragraph, "Written Description" requirement, 66 Fed. Reg. 1099 (Jan 5, 2001), a copy of which is attached hereto and filed herewith as Exhibit A.

Although the phrase "having unknown cellular proliferative activity" is not recited verbatim, Applicants assert that the specification would reasonably convey to one skilled in the relevant art that the inventor, at the time the application was filed, had possession of the claimed invention. Specifically, the specification states that the compound has an unknown effect in the assay of the present invention. The assays of the present invention are useful to determine the effect of a compound on cellular proliferation. The disclosure of the specification is sufficient through express, implicit, and inherent disclosure to support the patentability of Claims 1, 3-5, and 17-24 under the written description requirement of 35 U.S.C. § 112, first paragraph. Thus, Applicants assert that one of skill in the art could readily understand that the compounds to be tested have unknown cellular proliferative activity. Accordingly, Applicant respectfully requests that the rejection of Claims 1, 3-5, and 17-24 under 35 U.S.C. § 112, first paragraph, be withdrawn.

Claims 1, 3-5, and 17-24 are further rejected under 35 U.S.C. § 112, first paragraph, as failing to be enable one of skill in the art to make and use the invention. Specifically, the Examiner rejects Claim 21 due to the recitation the first cell culture and second cell culture are grown in serum-free media. Applicant asserts that Claim 21 does

sufficiently enable one of skill in the art to make and use the invention. Specifically, the specification of the above-identified application teaches one of ordinary skill in the art to:

(a) grow a first and second cell culture in serum-free media;

(b) add a compound to a first cell culture;

(c) measure the amount of HSPG in the first cell culture;

(d) compare the amount of HSPG in the first cell culture to the amount of HSPG in a second cell culture not treated with the compound; and

(e) determine if there is a change in the amount of HSPG in the first cell culture as compared to the amount of HSPG in the second cell culture.

Support and enablement for this method is found in Example VI.

The Examiner cites *Ettenson et al.* (J. Cell Physiol., 2000 Jul; 184(1):93-100) to support the contention that the method described above would not operate. However, as evidenced by Examples II-VI of the specification, this contention is not correct. Accordingly, Applicant respectfully asserts that this rejection under 35 U.S.C. § 112, first paragraph, is improper. Applicant refers the Examiner to Examples II-IV, each of which are performed using cell cultures grown in serum-free medium. These examples indicate that the amount or presence of HSPGs has a direct effect on smooth muscle cell growth, even in serum-free conditions. For example, perlecan inhibits smooth muscle cell proliferation in serum-free medium (Example II); perlecan mediates smooth muscle cell quiescence in serum-free conditions (Example III), and blocking perlecan synthesis leads to smooth muscle cell growth in serum-free medium. Accordingly, Applicant requests that the rejection of Claim 21 under 35 U.S.C. § 112, first paragraph, be withdrawn.

The Examiner further rejects Claims 1, 3-5, and 17-24 under 35 U.S.C. § 112, first paragraph, on the basis that the specification fails to disclose what type of cells produce syndecan or glypican, how to measure the amount of syndecan or glypican in cell culture, and how to discriminate between perlecan, syndecan, or glypican when measuring the amount of HSPGs. Applicant respectfully disagrees with each of these contentions. Applicant directs the Examiner to page 5, lines 28-30 of the specification which clearly states, "Examples of HSPGs include syndecan, glypican and perlecan which are HSPGs

generated within the cardiovascular system. Vascular SMCs express syndecans 1, 2 and 4, glypican-1 and perlecan." Further, Applicant points out that the discussion of perlecan in the specification is intended to be illustrative of the methods relating to HSPGs in general. Thus, Applicant asserts that upon reading how to immunoprecipitate perlecan using an anti-perlecan antibody for purposes of measurement, as indicated on page 13, lines 26-30, one of skill in the art would understand that a similar process could be used for the measurement of syndecan and glypican. In a related matter, immunoprecipitation of each HSPG would be a method by which one of skill in the art could discriminate between perlecan, syndecan, or glypican when measuring the amount of HSPGs.

Applicant notes that "[n]ot every last detail [of an invention need] be described [in a patent specification], else patent specifications would turn into production specifications, which they were never intended to be." *In re Gay*, 309 F.2d 769, 774, 135 U.S.P.Q. 311, 316 (C.C.P.A. 1962). Thus, although the specification does not describe the specifics of immunoprecipitation of syndecan and glypican, Applicant asserts that the immunoprecipitation of glypican and syndecan is "merely routine" and the working examples of perlecan measurement and isolation provide "a reasonable amount of guidance with respect to the direction in which the experimentation should proceed." *In re Wands,* 858 F.2d at 737, 8 U.S.P.Q.2d at 1404 (quoting *Ex parte Jackson*, 217 U.S.P.Q. 804, 807 (Bd. App. 1982)). Accordingly, Applicant respectfully requests that the rejection of Claims 1, 3-5, and 17-24 under 35 U.S.C. § 112, first paragraph, be withdrawn.

Finally, the Examiner rejects Claim 5 on the basis that the specification does not provide guidance to one of skill in the art as to what molecule is to be screened. Applicant restates the discussion of the case law presented above. Further, Applicant argues that the invention is not limited to any particular type of molecule, but rather, can encompass any molecule or compound. A person may use the methods of the present invention to determine the effect of <u>any</u> molecule or compound on cell proliferation. Thus, the identity of the molecule depends entirely on the inquiry performed by the end user of the invention. In light of the above discussion, Applicants assert that the claims of the present invention

cannot be limited to any particular class of molecules or any molecule having particular properties. Accordingly, Applicant respectfully requests that the rejection of Claim 5 under 35 U.S.C. § 112, first paragraph be withdrawn.

The Rejection Under 35 USC § 102

Claims 1, 3-5, 17, 19, and 22 are rejected under 35 U.S.C. § 102(b) as being anticipated by *Paka et al.* (Circulation, 1999 Nov.; 100(18, Suppl):I.548, abstract 2885) ("*Paka 1*"). Respectfully, the rejection is traversed.

The PTO states that *Paka 1* teaches "a method [to] determine whether the anti-proliferative effect of apoE is due to increased HS production." The PTO also states that *Paka 1* "concluded that the ability of apoE isoforms to inhibit SMC proliferation correlated with their ability to stimulate perlecan production," and thus, "[t]he reference teachings anticipate the claimed invention."

Paka 1 investigates the anti-proliferative effects of apoE, a well-known antiproliferative, on smooth muscle cells (SMCs). *Paka 1* discloses that apoE inhibits SMC proliferation and stimulates HSPG production. Finally, *Paka 1* suggests that the antiproliferative effect of apoE correlates with its ability to stimulate perlecan HSPGs and thus postulates that perlecan expression may be a key pathway in the regulation of SMC growth. *Paka 1* does not suggest a method for detecting further substances which affect cellular proliferation.

The invention as Claimed in Claim 1 is directed to a method for detecting a compound which affects cellular proliferation by adding the compound to a cell culture, measuring the amount of HSPG production, and comparing the amount of HSPG in the first cell culture to the amount of HSPG produced in a control cell culture. Similar methods are claimed in Claims 22-24, wherein the HSPG is perlecan, syndecan, or glypican, respectively.

As stated in the specification, "[a]lthough it is currently believed that endothelial HSPGs inhibit SMC proliferation, it is not known whether SMC synthesize antiproliferative HSPGs that act as autocrine inhibitors" (page 5, lines 21-23). While it was hypothesized that HSPGs may play a role in inhibition of cellular proliferation, the mechanism remained unclear. Only after the disclosure of Applicant's invention was it known that SMCs naturally regulate the induction of HSPGs to maintain quiescence (see for example, Examples I and IV). *Paka 1* does not disclose or suggest that cells naturally regulate and synthesize HSPGs that act as autocrine inhibitors. Thus, absent this significant piece of information, *Paka 1* does not reasonably teach or suggest a method for detecting compounds which affect cellular proliferation by measuring their effect on HSPG production. One of skill in the art would have to unduly experiment to discover that SMCs regulate cellular proliferation by inducing HSPG production. Thus, *Paka 1* does not teach or suggest the claimed invention. Accordingly, Applicant respectfully requests that the rejection of Claims 1, 3-5, 17, 19, and 22 under 35 U.S.C. § 102(b) be withdrawn.

Claims 1, 3-5, 17, 19, 20, 22, and 23 are rejected under 35 U.S.C. § 102(b) as being anticipated by *Paka et al.* (J. Biol. Chem, 1999 Dec.; 274(51):36403-36408) ("*Paka 2*"). Respectfully, the rejection is traversed.

The PTO states that *Paka 2* describes "a method [to] determine whether the anti-proliferative effect of apoE is due to increased HS production and cell growth." The PTO also states that *Paka 2* teaches "that analysis of the conditioned medium from apoE stimulated cells revealed that the HSPG increase was in perlecan and apoE also stimulated perlecan mRNA expression by > 2 fold."

Paka 2, much like *Paka 1*, investigates the anti-proliferative effects of apoE on SMCs. *Paka 2* discloses that apoE inhibits SMC proliferation and stimulates HSPG production (see abstract; Figs. 5 & 6). *Paka 2* states that *in vitro* isolated HSPGs are effective inhibitors of SMC proliferation (page 36407, col. 1, paragraph 1). *Paka 2* suggests that the antiproliferative effect of apoE correlates with its ability to stimulate perlecan

HSPGs (page 36406, first column, second paragraph). *Paka 2* also postulates that modulation of perlecan may be a key step in regulating SMC growth (page 36408, last paragraph). *Paka 2* does not suggest a method for detecting further substances which affect cellular proliferation.

Applicant restates the discussion above regarding *Paka 1*. Applicant further emphasizes that *Paka 2* does not disclose or suggest that cells naturally regulate and synthesize HSPGs that act as autocrine inhibitors. Again, absent this significant piece of information, *Paka 2* cannot reasonably teach or suggest a method for detecting compounds which affect cellular proliferation by measuring their effect on HSPG production. Thus, *Paka 2* does not teach or suggest the claimed invention. Accordingly, Applicant respectfully requests that the rejection of Claims 1, 3-5, 17, 19, 20, 22, and 23 under 35 U.S.C. § 102(b) be withdrawn.

Claims 1, 3-5, 17, 19, 20, and 22 are rejected under 35 U.S.C. § 102(b) as being anticipated by *Obunike et al.* (Arter. Thromb. Vasc. Biol., 2000 Jan.; 20(1):111-118) ("*Obunike*") as evidenced by *Paka 2*. Respectfully, the rejection is traversed.

The PTO states that *Obunike* teaches "a method to test whether the expression of apoE or LPL would modulate protegylcan (PG) metabolism in cells." The PTO also states that *Obunike* "concluded that enhanced gene expression of apoE and LPL increases cellular PG expression," and thus, "[t]he reference teachings anticipate the claimed invention."

Obunike investigates the effects of apoE on proteoglycan production. *Obunike* discloses that apoE and LPL bind with high affinity to HSPGs (see abstract). *Obunike* observes that both apoE and LPL increase PG synthesis or decrease PG degradation (page 113, col. 1, last paragraph; Figure 3). *Obunike* does not suggest a method for detecting further substances which affect cellular proliferation.

Applicant restates the discussion above as it relates to *Paka 1* and *Paka 2*. Again, only after the disclosure of Applicant's invention was it known that SMCs naturally regulate the

induction of HSPGs to maintain quiescence. Thus, while it was hypothesized that HSPGs may play a role in inhibition of cellular proliferation, the mechanism remained unclear. *Obunike* does not disclose or suggest that cells naturally regulate and synthesize HSPGs that act as autocrine inhibitors. Further, as discussed above, *Paka 2* also fails to disclose or suggest that cells naturally regulate and synthesize HSPGs that act as autocrine inhibitors. Absent this significant piece of information, neither *Obunike* nor *Paka 2*, either alone or in combination, reasonably teach or suggest a method for detecting compounds which affect cellular proliferation by measuring their effect on HSPG production. Accordingly, Applicant respectfully requests withdrawal of the rejection of Claims 1, 3-5, 17, 19, 20, and 22 under 35 U.S.C. § 102(b).

The Rejection Under 35 USC § 103

Claims 1, 3, and 23 are rejected under 35 U.S.C. § 103(a) as being unpatentable over *Paka 1* or *Obunike* in view of *Paka 2*. Respectfully, this rejection is traversed. As discussed above, neither *Paka 1*, *Paka 2*, or *Obunike* describe or suggest that SMCs naturally regulate the induction of HSPGs to maintain quiescence and act as autocrine inhibitors. Absent this significant piece of information, *Paka 1*, *Paka 2*, or *Obunike*, either alone or in combination, do not reasonably suggest a method for detecting compounds which affect cellular proliferation by measuring their effect on HSPG production. Accordingly, Applicant respectfully requests that the rejection of Claims 1, 3, and 23 under 35 U.S.C. § 103(a) be withdrawn.

Claims 1, 3, and 24 are rejected under 35 U.S.C. § 103(a) as being unpatentable over *Paka 1* or *Paka 2* or *Obunike* in view of U.S. Patent No. 6,306,613 to *Florkiewicz et al.* ("*Florkiewicz*"). Applicant respectfully traverses this rejection. *Florkiewicz* is cited to support the Examiner's contention that glypican is known to play a role in cellular proliferation. Applicant disagrees with this contention. Applicant further notes that the filing date of *Florkiewicz* is October 23, 2001, which does not antedate the March 2, 2001 priority date of the present application, and thus, cannot properly be applied

as prior art under 35 U.S.C. § 103(a). Further, Applicants assert that the subject matter relating to the role of glypican in cellular proliferation is not entitled to the earlier filing dates of either U.S.S.N. 09/030,613 or U.S.S.N. 08/807,014. Neither U.S.S.N. 09/030,613 nor U.S.S.N. 08/807,014. make any reference to the role of glypican in cellular proliferation. Accordingly, such information constitutes new matter and is entitled only to the filing date of October 23, 2001.

MPEP § 2136.03 states that

> In order to carry back the 35 U.S.C. 102(e) critical date of the U.S. patent reference to the filing date of a parent application, the U.S. patent reference must have a right of priority to the earlier date under 35 U.S.C. 120 or 365(c) and the parent application must support the invention claimed as required by 35 U.S.C. 112, first paragraph. "For if a patent could not theoretically have issued the day the application was filed, it is not entitled to be used against another as 'secret prior art'" under 35 U.S.C. 102(e). *In re Wertheim*, 646 F.2d 527, 537, 209 USPQ 554, 564 (CCPA 1981).

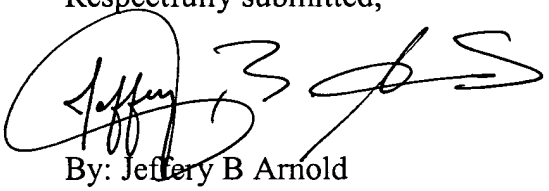
Accordingly, Applicant respectfully requests that the rejection of Claims 1, 3, and 24 under 35 U.S.C. § 103(a) be withdrawn.

CONCLUSION

In view of the above remarks, Applicant respectfully asserts that the rejection of the claims as set forth in the Office Action has been addressed and overcome. Applicant further respectfully asserts that all claims are in condition for allowance and requests that an early notice of allowance be issued. If issues may be resolved through Examiner's Amendment, or clarified in any manner, a call to the undersigned attorney at (404) 879-2433 is respectfully requested.

No fees are believed due, however, the Commissioner if hereby authorized to charge any deficiencies which may be required, or credit any overpayment to Deposit Account No. 09-0528.

Respectfully submitted,



By: Jeffery B Arnold
Reg. No. 39,540

WOMBLE CARLYLE SANDRIDGE & RICE, PLLC
P.O. Box 7037
Atlanta, Georgia 30357-0037
Direct Telephone: (404) 879-2433
Direct Facsimile: (404) 879-2933
Firm Telephone: (404) 872-7000

Atty. Docket No.: R18631 1050.1 (R18631-0141) (51880.0010.9)

EXHIBIT A

an asserted utility, unless countervailing evidence can be provided that shows that one of ordinary skill in the art would have a legitimate basis to doubt the credibility of such a statement. Similarly, Office personnel must accept an opinion from a qualified expert that is based upon relevant facts whose accuracy is not being questioned; it is improper to disregard the opinion solely because of a disagreement over the significance or meaning of the facts offered.

Once a *prima facie* showing of no specific and substantial credible utility has been properly established, the applicant bears the burden of rebutting it. The applicant can do this by amending the claims, by providing reasoning or arguments, or by providing evidence in the form of a declaration under 37 CFR 1.132 or a patent or a printed publication that rebuts the basis or logic of the *prima facie* showing. If the applicant responds to the *prima facie* rejection, the Office personnel should review the original disclosure, any evidence relied upon in establishing the *prima facie* showing, any claim amendments, and any new reasoning or evidence provided by the applicant in support of an asserted specific and substantial credible utility. It is essential for Office personnel to recognize, fully consider and respond to each substantive element of any response to a rejection based on lack of utility. Only where the totality of the record continues to show that the asserted utility is not specific, substantial, and credible should a rejection based on lack of utility be maintained.

If the applicant satisfactorily rebuts a *prima facie* rejection based on lack of utility under § 101, withdraw the § 101 rejection and the corresponding rejection imposed under § 112, first paragraph.

Dated: December 29, 2000.

Q. Todd Dickinson,

Under Secretary of Commerce for Intellectual Property and Director of the United States Patent and Trademark Office.

[FR Doc. 01–322 Filed 1–4–01; 8:45 am]

BILLING CODE 3510–16–U

DEPARTMENT OF COMMERCE

United States Patent and Trademark Office

[Docket No. 991027288–0264–02]

RIN 0651–AB10

Guidelines for Examination of Patent Applications Under the 35 U.S.C. 112, ¶ 1, "Written Description" Requirement

AGENCY: United States Patent and Trademark Office, Commerce.

ACTION: Notice.

SUMMARY: These Guidelines will be used by USPTO personnel in their review of patent applications for compliance with the "written description" requirement of 35 U.S.C. 112, ¶ 1. These Guidelines supersede the "Revised Interim Guidelines for Examination of Patent Applications Under the 35 U.S.C. 112, ¶ 1 'Written Description' Requirement" that were published in the **Federal Register** at 64 FR 71427, Dec. 21, 1999, and in the Official Gazette at 1231 O.G. 123, Feb. 29, 2000. These Guidelines reflect the current understanding of the USPTO regarding the written description requirement of 35 U.S.C. 112, ¶ 1, and are applicable to all technologies.

DATES: The Guidelines are effective as of January 5, 2001.

FOR FURTHER INFORMATION CONTACT: Stephen Walsh by telephone at (703) 305–9035, by facsimile at (703) 305–9373, by mail to his attention addressed to United States Patent and Trademark Office, Box 8, Washington, DC 20231, or by electronic mail at "stephen.walsh@uspto.gov"; or Linda Therkorn by telephone at (703) 305–8800, by facsimile at (703) 305–8825, by mail addressed to Box Comments, Commissioner for Patents, Washington, DC 20231, or by electronic mail at "linda.therkorn@uspto.gov."

SUPPLEMENTARY INFORMATION: As of the publication date of this notice, these Guidelines will be used by USPTO personnel in their review of patent applications for compliance with the "written description" requirement of 35 U.S.C. 112, ¶ 1. Because these Guidelines only govern internal practices, they are exempt from notice and comment rulemaking under 5 U.S.C. 553(b)(A).

Discussion of Public Comments

Comments were received from 48 individuals and 18 organizations in response to the request for comments on the "Revised Interim Guidelines for Examination of Patent Applications Under the 35 U.S.C. 112, ¶ 1 'Written Description' Requirement" published in the **Federal Register** at 64 FR 71427, Dec. 21, 1999, and in the Official Gazette at 1231 O.G. 123, Feb. 29, 2000. The written comments have been carefully considered.

Overview of Comments

The majority of comments favored issuance of final written description guidelines with minor revisions. Comments pertaining to the written description guidelines are addressed in detail below. A few comments addressed particular concerns with respect to the associated examiner training materials that are available for public inspection at the USPTO web site (www.uspto.gov). Such comments will be taken under advisement in the revision of the training materials; consequently, these comments are not specifically addressed below as they do not impact the content of the Guidelines. Several comments raised issues pertaining to the patentability of ESTs, genes, or genomic inventions with respect to subject matter eligibility (35 U.S.C. 101), novelty (35 U.S.C. 102), or obviousness (35 U.S.C. 103). As these comments do not pertain to the written description requirement under 35 U.S.C. 112, they have not been addressed. However, the aforementioned comments are fully addressed in the "Discussion of Public Comments" in the "Utility Examination Guidelines" Final Notice, which will be published at or about the same time as the present Guidelines.

Responses to Specific Comments

(1) *Comment:* One comment stated that the Guidelines instruct the patent examiner to determine the correspondence between what applicant has described as the essential identifying characteristic features of the invention and what applicant has claimed, and that such analysis will lead to error. According to the comment, the examiner may decide what applicant should have claimed and reject the claim for failure to claim what the examiner considers to be the invention. Another comment suggested that the Guidelines should clarify what is meant by "essential features of the invention." Another comment suggested that what applicant has identified as the "essential distinguishing characteristics" of the invention should be understood in terms of *Fiers* v. *Revel*, 984 F.2d 1164, 1169, 25 USPQ2d 1601, 1605 (Fed. Cir. 1993) ("Conception of a substance claimed *per se* without reference to a process requires conception of its structure, name,

formula, or definitive chemical or physical properties.").

Response: The suggestions have been adopted in part. The purpose of the written description analysis is to confirm that applicant had possession of what is claimed. The Guidelines have been modified to instruct the examiners to compare the scope of the invention claimed with the scope of what applicant has defined in the description of the invention. That is, the Guidelines instruct the examiner to look for consistency between a claim and what provides adequate factual support for the claim as judged by one of ordinary skill in the art from reading the corresponding written description.

(2) *Comment:* Two comments urge that *Regents of the University of California* v. *Eli Lilly & Co.*, 119 F.3d 1559, 43 USPQ2d 1398 (Fed. Cir. 1997), is bad law and should not be followed by the USPTO because it conflicts with binding precedent, such as *Vas-Cath* v. *Mahurkar*, 935 F.2d 1555, 19 USPQ2d 1111 (Fed. Cir. 1991). *Response:* The final Guidelines are based on the Office's current understanding of the law and are believed to be fully consistent with binding precedent of the U.S. Supreme Court and the U.S. Court of Appeals for the Federal Circuit. *Eli Lilly* is a precedential decision by the Court that has exclusive jurisdiction over appeals involving patent law. Accordingly, the USPTO must follow *Eli Lilly*. Furthermore, the USPTO does not view *Eli Lilly* as conflicting with *Vas-Cath*. *Vas-Cath* explains that the purpose of the written description requirement is to ensure that the applicant has conveyed to those of skill in the art that he or she was in possession of the claimed invention at the time of filing. *Vas-Cath*, 935 F.2d at 1563–64, 19 USPQ2d at 1117. *Eli Lilly* explains that a chemical compound's name does not necessarily convey a written description of the named chemical compound, particularly when a genus of compounds is claimed. *Eli Lilly*, 119 F.3d at 1568, 43 USPQ2d at 1405. The name, if it does no more than distinguish the claimed genus from all others by function, does not satisfy the written description requirement because "it does not define any structural features commonly possessed by members of the genus that distinguish them from others. One skilled in the art therefore cannot, as one can do with a fully described genus, visualize or recognize the identity of the members of the genus." *Eli Lilly*, 119 F.3d at 1568, 43 USPQ2d at 1406. Thus, *Eli Lilly* identified a set of circumstances in which the words of the claim did not, without more, adequately convey to others that applicants had possession of what they claimed.

(3) *Comment:* Several comments urged that the Guidelines do not recognize the inconsistency between the original claim doctrine and the written description requirement as set out in *Fiers* and *Eli Lilly*. On the other hand, another comment asserts that there is no strong presumption that an originally filed claim constitutes an adequate written description of the claimed subject matter. Several comments indicate that *in haec verba* support should be sufficient to comply with the written description requirement. Two comments urge that the concept of constructive reduction to practice upon filing of an application has been ignored. *Response:* As noted above, the USPTO does not find *Fiers* and *Eli Lilly* to be in conflict with binding precedent. An original claim may provide written description for itself, but it still must be an *adequate* written description which establishes that the inventor was in possession of the invention. The "original claim doctrine" is founded on cases which stand for the proposition that originally filed claims are part of the written description of an application as filed, and thus subject matter which is present only in originally filed claims need not find independent support in the specification. *See, e.g., In re Koller*, 613 F.2d 819, 824, 204 USPQ 702, 706 (CCPA 1980) (later added claims of similar scope and wording were adequately described by original claims); *In re Gardner*, 480 F.2d 879, 880, 178 USPQ 149, 149 (CCPA 1973) ("Under these circumstances, we consider the original claim in itself adequate 'written description' of the claimed invention. It was equally a 'written description' * * * whether located among the original claims or in the descriptive part of the specification."). However, as noted in the preceding comment, *Eli Lilly* identified a set of circumstances in which the words of the claim did not, without more, adequately convey to others that applicants had possession of what they claimed. When the name of a novel chemical compound does not convey sufficient structural information about the compound to identify the compound, merely reciting the name is not enough to show that the inventor had possession of the compound at the time the name was written. The Guidelines indicate that there is a "strong presumption" that an adequate written description of the claimed invention is present when the application is filed, consistent with *In re Wertheim*, 541 F.2d 257, 263, 191 USPQ 90, 97 (CCPA 1976) ("we are of the opinion that the PTO has the initial burden of presenting evidence or reasons why persons skilled in the art would not recognize in the disclosure a description of the invention defined by the claims."). In most cases, the statement that "an originally filed claim is its own written description," is borne out because the claim language conveys to others of skill in the art that the applicant was "in possession" of what is claimed. The Guidelines emphasize that the burden of proof is on the examiner to establish that a description as filed is not adequate and require the examiner to introduce sufficient evidence or technical reasoning to shift the burden of going forward with contrary evidence to the applicant.

(4) *Comment:* One comment stated that the Guidelines change the substance of the written description requirement to require some level of enablement. The comment stated that the *Eli Lilly* case should not be followed because its change in the quality of the description required is in conflict with precedent. Another comment suggested that to comply with the written description requirement, the description must both (i) demonstrate possession of the claimed invention by the applicant; and (ii) put the public in possession of the claimed invention. *Response:* As noted in the comment above, the USPTO is bound by the Federal Circuit's decision in *Eli Lilly*. The Guidelines have been revised to clarify that an applicant must provide a description of the claimed invention which shows that applicant was in possession of the claimed invention. The suggestion to emphasize that the written description requirement must put the public in possession of the invention has not been adopted because it removes much of the distinction between the written description requirement and the enablement requirement. Although the two concepts are entwined, they are distinct and each is evaluated under separate legal criteria. The written description requirement, a question of fact, ensures that the inventor conveys to others that he or she had possession of the claimed invention; whereas, the enablement requirement, a question of law, ensures that the inventor conveys to others how to make and use the claimed invention.

(5) *Comment:* One comment suggested that the Guidelines should provide examples of situations in which the written description requirement was met but the enablement requirement was not, and vice versa. Another comment stated that examiners often use enablement language in making

written description rejections. *Response:* The enablement and written description requirements are not coextensive and, therefore, situations will arise in which one requirement is met but the other is not. Federal Circuit case law demonstrates many circumstances where enablement or written description issues, but not both, were before the Court. These Guidelines are intended to clarify for the examining corps the criteria needed to satisfy the written description requirement. For examples applying these Guidelines to hypothetical fact situations, see the "Synopsis of Application of Written Description Guidelines" (examiner training materials available on-line at *http://www.uspto.gov/web/menu/written.pdf*). These examples, as well as the examination form paragraphs and instructions on their proper use, provide the appropriate language examiners should use in making written description rejections.

(6) *Comment:* One comment disagreed with the statement in an endnote that "the fact that a great deal more than just a process is necessary to render a product invention obvious means that a great deal more than just a process is necessary to provide written description for a product invention." The comment indicated that the statement is overly broad and inconsistent with the "strong presumption that an adequate written description of the claimed invention is present when the application is filed." As an extreme case, for example, for product-by-process claims, nothing else would be needed to provide the written description of the product. *Response:* The endnote has been clarified and is now more narrowly drawn. However, there is no *per se* rule that disclosure of a process is sufficient to adequately describe the products produced by the process. In fact, *Fiers* v. *Revel* and *Eli Lilly* involved special circumstances where the disclosure of a process of making and the function of the product alone did not provide an adequate written description for product claims. Even when a product is claimed in a product-by-process format, the adequacy of the written description of the process to support product claims must be evaluated on a case-by-case basis.

(7) *Comment:* Several comments urge that actual reduction to practice, as a method of satisfying the written description requirement by demonstrating possession, has been over-emphasized. *Response:* The Guidelines have been clarified to state that describing an actual reduction to practice is one of a number of ways to show possession of the invention. Description of an actual reduction to practice offers an important "safe haven" that applies to all applications and is just one of several ways by which an applicant may demonstrate possession of the claimed invention. Actual reduction to practice may be crucial in the relatively rare instances where the level of knowledge and level of skill are such that those of skill in the art cannot describe a composition structurally, or specify a process of making a composition by naming components and combining steps, in such a way as to distinguish the composition with particularity from all others. Thus, the emphasis on actual reduction to practice is appropriate in those cases where the inventor cannot provide an adequate description of what the composition is, and a definition by function is insufficient to define a composition "because it is only an indication of what the [composition] does, rather than what it is." *Eli Lilly*, 119 F.3d at 1568, 43 USPQ at 1406. *See also Amgen Inc.* v. *Chugai Pharmaceutical Co.*, 927 F.2d 1200, 1206, 18 USPQ2d 1016, 1021 (Fed. Cir. 1991).

(8) *Comment:* One comment asserts that the citation to *Pfaff* v. *Wells Electronics, Inc.*, 525 U.S. 55, 48 USPQ2d 1641 (1998) is inappropriate and should be deleted because *Pfaff* is concerned with § 102(b) on-sale bar, not written description. Another comment suggested that the Guidelines should provide an explanation of how the "ready for patenting" concept of *Pfaff* should be used in determining compliance with the written description requirement. *Response:* The Guidelines state the general principle that actual reduction to practice is not required to show possession of, or to adequately describe, a claimed invention (although, as noted in the previous comment, an actual reduction to practice is crucial in relatively rare instances). An alternative is to show that the invention described was "ready for patenting" as set out in *Pfaff*. For example, a *description* of activities that demonstrates the invention was "ready for patenting" satisfies the written description requirement. As *Wertheim* indicates, "how the specification accomplishes this is not material." 541 F.2d at 262, 191 USPQ at 96.

(9) *Comment:* One comment stated that the written description of a claimed DNA should be required to include the complete sequence of the DNA and claims should be limited to the DNA sequence disclosed. *Response:* Describing the complete chemical structure, *i.e.*, the DNA sequence, of a claimed DNA is one method of satisfying the written description requirement, but it is not the only method. *See Eli Lilly*, 119 F.3d at 1566, 43 USPQ2d at 1404 ("An adequate written description of a DNA * * * requires a precise definition, *such as* by structure, formula, chemical name, or physical properties." (emphasis added, internal quote omitted)). Therefore, there is no basis for a *per se* rule requiring disclosure of complete DNA sequences or limiting DNA claims to only the sequence disclosed.

(10) *Comment:* One comment stated that it is difficult to envision how one could provide a description of sufficient identifying characteristics of the invention without physical possession of a species of the invention, and thus this manner of showing possession should be considered as a way to show actual reduction to practice. *Response:* This suggestion has not been adopted. The three ways of demonstrating possession as set forth in the Guidelines are merely exemplary and are not mutually exclusive. While there are some cases where a description of sufficient relevant identifying characteristics will evidence an actual reduction to practice, there are other cases where it will not. *See, e.g., Ralston Purina Co.* v. *Far-Mar-Co, Inc.*, 772 F.2d 1570, 1576, 227 USPQ 177, 180 (Fed. Cir. 1985) (disclosure taken with the knowledge of those skilled in the art may be sufficient support for claims).

(11) *Comment:* One comment stated that the Guidelines should be revised to indicate that the test of disclosure of sufficiently detailed drawings should be expanded to include structural claiming of chemical entities. *Response:* The suggestion has been adopted.

(12) *Comment:* One comment stated that the Guidelines should reflect that an inventor is in possession of the invention when the inventor demonstrably has at least a complete conception thereof, and that factors and attributes which provide proof of written description should include evidence typically provided to prove a complete conception. *Response:* The suggestion has not been adopted because the conception analysis typically involves documentary evidence in addition to the description of the invention in the application as filed. However, it is acknowledged that if evidence typically provided to prove a complete conception is present in the specification as filed, it would be sufficient to show possession. The Federal Circuit has stated "[t]he conception analysis necessarily turns on the inventor's ability to describe his invention with particularity. Until he can do so, he cannot prove possession

of the complete mental picture of the invention." *Burroughs Wellcome Co.* v. *Barr Labs., Inc.*, 40 F.3d 1223, 1228, 32 USPQ2d 1915, 1919 (Fed. Cir. 1994). As further noted by the Federal Circuit, in order to prove conception, "a party must show possession of every feature recited in the count, and that every limitation of the count must have been known to the inventor at the time of the alleged conception." *Coleman* v. *Dines*, 754 F.2d 353, 359, 224 USPQ 857, 862 (Fed. Cir. 1985).

(13) *Comment:* One comment indicated that a "possession" test does not appear in Title 35 of the U.S. Code and is not clearly stated by the Federal Circuit. Therefore, it is recommended that patent examiners be directed to use existing judicial precedent to make rejections of claims unsupported by a statutory written description requirement. *Response:* While the Federal Circuit has not specifically laid out a "possession" test, the Court has clearly indicated that possession is a cornerstone of the written description inquiry. *See, e.g., Vas-Cath, Inc.* v. *Mahurkar*, 935 F.2d 1555, 1563, 19 USPQ2d 1111, 1116 (Fed. Cir. 1991); *see also Purdue Pharma L.P.* v. *Faulding Inc.*, 230 F.3d 1320, 1323, 56 USPQ2d 1481, 1483 (Fed. Cir. 2000) ("[o]ne skilled in the art, reading the disclosure, must immediately discern the limitation at issue in the claims") (internal quote omitted). The possession test as set forth in the Guidelines is extrapolated from case law in a wide variety of technologies and is not intended to be limiting. Any rejections made by examiners will be made under 35 U.S.C. 112, ¶1, with supporting rationale. Final rejections are appealable if applicant disagrees and follows the required procedures to appeal.

(14) *Comment:* Two comments indicated that if the amino acid sequence for a polypeptide whose utility has been identified is described, then the question of possession of a class of nucleotides encoding that polypeptide can be addressed as a relatively routine matter using the understanding of the genetic code, and that the endnote addressing this issue should be revised. *Response:* The suggestion of these comments has been incorporated in the Guidelines and will be reflected in the training materials. However, based upon *In re Bell*, 991 F.2d 781, 785, 26 USPQ2d 1529, 1532 (Fed. Cir. 1993) and *In re Baird*, 16 F.3d 380, 382, 29 USPQ2d 1550, 1552 (Fed. Cir. 1994), this does not mean that applicant was in possession of any particular species of the broad genus.

(15) *Comment:* One comment disagreed with an endnote which stated that a laundry list disclosure of moieties does not constitute a written description of every species in a genus. Specifically, the comment indicates that if the existence of a functional genus is adequately described in the specification, a laundry list of the species within that genus must satisfy the written description requirement. *Response:* The suggestion to revise the endnote will not be adopted. A lack of adequate written description problem arises if the knowledge and level of skill in the art would not permit one skilled in the art to immediately envisage the product claimed from the disclosure. This was aptly demonstrated in *In re Bell* and *In re Baird* where possession of a large genus did not put a person of ordinary skill in the art in possession of any particular species. *See also Purdue Pharma*, 230 F.3d at 1328, 56 USPQ2d at 1487 (because the original specification did not disclose the later claimed concentration ratio was a part of the invention, the inventors cannot argue that they are merely narrowing a broad invention).

(16) *Comment:* One comment suggested that in the majority of cases, a single species will support a generic claim, and that the Guidelines should emphasize this point. *Response:* The suggestion has been adopted to a limited degree. The Guidelines now indicate that a single species may, in some instances, provide an adequate written description of a generic claim when the description of the species would evidence to one of ordinary skill in the art that the invention includes the genus. Note, however, *Tronzo* v. *Biomet, Inc.*, 156 F.3d 1154, 47 USPQ2d 1829 (Fed. Cir. 1998), where the species in the parent application was held not to provide written description support for the genus in the child application.

(17) *Comment:* One comment asserted that the Guidelines should focus on the compliance of the claims, not the specification, with the written description requirement. *Response:* This suggestion will not be adopted. "The specification shall contain a written description of the invention." 35 U.S.C. 112. The claims are part of the specification. *Id.*, ¶ 2. If an adequate description is provided, it will suffice "whether located among the original claims or in the descriptive part of the specification." *In re Gardner*, 480 F.2d 879, 880, 178 USPQ 149 (CCPA 1973). The entire disclosure, including the specification, drawings, and claims, must be considered.

(18) *Comment:* One comment asserted that the Guidelines confuse "new matter," 35 U.S.C. 132, with the written description requirement, and that the same standard for written description should be applied to both original claims and new or amended claims. *Response:* The Guidelines indicate that for both original and amended claims, the inquiry is whether one skilled in the art can reasonably conclude that the inventor had possession of the claimed invention at the time the application was filed.

(19) *Comment:* One comment suggested that the second paragraph of the section pertaining to determining what the claim as a whole covers should be deleted because it relates more to compliance with § 112, second paragraph, than with the written description requirement. *Response:* This suggestion will not be adopted. The claims must be construed and all issues as to the scope and meaning of the claim must be explored during the inquiry into whether the written description requirement has been met. The concept of treating the claim as a whole is applicable to all criteria for patentability.

(20) *Comment:* One comment suggested a different order for the general analysis for determining compliance with the written description requirement, starting with reading the claim, then the specification, and then determining whether the disclosure demonstrates possession by the applicant. *Response:* This suggestion will not be adopted. The claims must be construed as broadly as reasonable in light of the specification and the knowledge in the art. *See In re Morris*, 127 F.3d 1048, 1054, 44 USPQ2d 1023, 1027 (Fed. Cir. 1997). Then the disclosure must be evaluated to determine whether it adequately describes the claimed invention, i.e., whether it conveys to a person having ordinary skill in the art that the applicant had possession of what he or she now claims.

(21) *Comment:* Several comments suggested that the Guidelines are unclear with regard to how the examiner should treat the transitional phrase "consisting essentially of." The comments also suggested that the endnote that explains "consisting essentially of" does not make clear how the use of this intermediate transitional language affects the scope of the claim. Several comments stated that the USPTO does not have legal authority to treat claims reciting this language as open (equivalent to "comprising"). Another comment suggested that the phrase "clear indication in the specification" be replaced with "explicit or implicit indication." *Response:* The transitional phrase "consisting essentially of" "excludes

ingredients that would 'materially affect the basic and novel characteristics' of the claimed composition." *Atlas Powder Co.* v. *E.I. DuPont de Nemours & Co.*, 750 F.2d 1569, 1574, 224 USPQ 409, 412 (Fed. Cir. 1984). The basic and novel characteristics of the claimed invention are limited by the balance of the claim. *In re Janakirama-Rao*, 317 F.2d 951, 954, 137 USPQ 893, 896 (CCPA 1963). However, during prosecution claims must be read broadly, consistent with the specification. *In re Morris*, 127 F.3d 1048, 1054, 44 USPQ2d 1023, 1027 (Fed. Cir. 1997). Thus, *for purposes of searching for and applying prior art in a rejection under 35 U.S.C. 102 or 103*, if the specification or the claims do not define the "basic and novel" properties of the claimed subject matter (or if such properties are in dispute), the broadest reasonable interpretation consistent with the specification is that the basic and novel characteristics are merely the presence of the recited limitations. *See, e.g., Janakirama-Rao*, 317 F.2d at 954, 137 USPQ at 895–96. This does not indicate that the intermediate transitional language is never given weight. Applicants may amend the claims to avoid the rejections or seek to establish that the specification provides definitions of terms in the claims that define the basic and novel characteristics of the claimed invention which distinguish the claimed invention from the prior art. When an applicant contends that additional steps or materials in the prior art are excluded by the recitation of 'consisting essentially of,' applicant has the burden of showing that the introduction of additional steps or components would materially change the characteristics of applicant's invention. *In re De Lajarte*, 337 F.2d 870, 143 USPQ 256 (CCPA 1964). The language used in the Guidelines is consistent with *PPG Industries Inc.* v. *Guardian Industries Corp.*, 156 F.3d 1351, 1355, 48 USPQ2d 1351, 1355 (Fed. Cir. 1998) ("PPG could have defined the scope of the phrase 'consisting essentially of' for purposes of its patent by making clear in its specification what it regarded as constituting a material change in the basic and novel characteristics.").

(22) *Comment:* One comment stated that the written description should "disclose the invention," including why the invention works and how it was developed. *Response:* This suggestion has not been adopted. An inventor does not need to know how or why the invention works in order to obtain a patent. *Newman* v. *Quigg*, 877 F.2d 1575, 1581, 11 USPQ2d 1340, 1345 (Fed. Cir. 1989). To satisfy the enablement requirement of 35 U.S.C. 112, ¶1, an application must disclose the claimed invention in sufficient detail to enable a person of ordinary skill in the art to make and use the claimed invention. To satisfy the written description requirement of 35 U.S.C. 112, ¶ 1, the description must show that the applicant was in possession of the claimed invention at the time of filing. There is no statutory basis to require disclosure of why an invention works or how it was developed. "Patentability shall not be negatived by the manner in which the invention was made." 35 U.S.C. 103(a).

(23) *Comment:* One comment recommended that the phrases "emerging and unpredictable technologies" and "unpredictable art" be replaced with the phrase—inventions characterized by factors which are not reasonably predictable in terms of the ordinary skill in the art—. *Response:* The suggestion is adopted in part and the recommended phrase has been added as an alternative.

(24) *Comment:* One comment recommended that the phrase "conventional in the art" be replaced with—part of the knowledge of one of ordinary skill in the art—. *Response:* The suggestion is adopted in part and the recommended phrase has been added as an alternative. The standard of "conventional in the art" is supported by case law holding that a patent specification "need not teach, and preferably omits, what is well known in the art." *See Spectra-Physics, Inc.* v. *Coherent, Inc.*, 827 F.2d 1524, 1534, 3 USPQ2d 1737, 1743 (Fed. Cir. 1987); *Hybritech Inc.* v. *Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 1384, 231 USPQ 81, 94 (Fed. Cir. 1986). *See also Atmel Corp.* v. *Information Storage Devices, Inc.*, 198 F.3d 1374, 1382, 53 USPQ2d 1225, 1231 (Fed. Cir. 1999).

(25) *Comment:* One comment recommended that the Guidelines be amended to state that the appropriate skill level for determining possession of the claimed invention is that of a person of *ordinary* skill in the art. *Response:* The comment has not been adopted. The statutory language itself indicates that compliance with the requirements of 35 U.S.C. 112, ¶1, is judged from the standard of "any person skilled in the art." It is noted, however, that the phrases "one of skill in the art" and "one of ordinary skill in the art" appear to be synonymous. *See, e.g., Union Oil Co.* v. *Atlantic Richfield Co.*, 208 F.3d 989, 997, 54 USPQ2d 1227, 1232 (Fed. Cir. 2000) ("The written description requirement does not require the applicant 'to describe exactly the subject matter claimed, [instead] the description must clearly allow *persons of ordinary skill in the art* to recognize that [he or she] invented what is claimed.' Thus, § 112, ¶ 1, ensures that, as of the filing date, the inventor conveyed with reasonable clarity to *those of skill in the art* that he was in possession of the subject matter of the claims." (citations omitted, emphasis added)).

(26) *Comment:* One comment stated that an endnote misstates the relevant law in stating that, to show inherent written descriptive support for a claim limitation, the inherent disclosure must be such as would be recognized by a person of ordinary skill in the art. The comment recommended that the endnote be amended to delete the reference to recognition by persons of ordinary skill and to cite *Pingree* v. *Hull*, 518 F.2d 624, 186 USPQ 248 (CCPA 1975), rather than *In re Robertson*, 169 F.3d 743, 49 USPQ2d 1949 (Fed. Cir. 1999). *Response:* The comment has not been adopted. Federal Circuit precedent makes clear that an inherent disclosure must be recognized by those of ordinary skill in the art. *See, e.g., Hyatt* v. *Boone*, 146 F.3d 1348, 1354–55, 47 USPQ2d 1128, 1132 (Fed. Cir. 1998) ("[T]he purpose of the description requirement is 'to ensure that the inventor had possession, as of the filing date of the application relied on, of the specific subject matter later claimed by him.' * * * Thus, the written description must include all of the limitations of the interference count, or the applicant must show that any absent text is necessarily comprehended in the description provided *and would have been so understood* at the time the patent application was filed." (emphasis added)). *See also Reiffin* v. *Microsoft Corp.*, 214 F.3d 1342, 1346, 54 USPQ2d 1915, 1917 (Fed. Cir. 2000) (The "application considered as a whole must convey to one of ordinary skill in the art, either explicitly or inherently, that [the inventor] invented the subject matter claimed * * *. See * * * *Continental Can Co. USA* v. *Monsanto Co.*, 948 F.2d 1264, 1268, 20 USPQ2d 1746, 1749 (Fed. Cir. 1991) (descriptive matter may be inherently present in a specification if one skilled in the art would necessarily recognize such a disclosure)").

(27) *Comment:* Several comments pointed out an inconsistency in the **Federal Register** Notice re: the Revised Interim Written Description Guidelines. The inconsistency concerned the treatment of claims directed to an isolated DNA comprising SEQ ID NO:1 wherein SEQ ID NO:1 is an expressed sequence tag. The comments contrasted paragraphs 34 and 35 of the Response to

Public Comments with the statement in the text of the Guidelines that a genus must be supported by a representative number of species (as analyzed in Example 7 of the training materials). *Response:* The USPTO acknowledges that there was an inconsistency. The Office notes that a claim reciting a nucleic acid comprising SEQ ID NO:1 may be subject to a rejection for lack of an adequate written description where particular identifiable species within the scope of the claim lack an adequate written description. The training materials as amended exemplify an appropriate analysis.

(28) *Comment:* One comment stated that the USPTO should respond to the issue of whether the U.S. is meeting its TRIPs obligations. This comment noted that the USPTO did not address an earlier comment regarding the "Interim Guidelines for the Examination of Patent Applications under the 35 U.S.C. 112, ¶ 1, 'Written Description' Requirement," 63 FR 32,639, June 15, 1998, which questioned whether the written description requirement is truly different from the enablement requirement, and indicated that such a requirement may be contrary to the TRIPs provisions of the World Trade Organization (Article 27.1). Article 27.1 requires WTO Members to, *inter alia*, make patents available, with limited exceptions, for products and processes in all fields of technology so long as those products and processes are new, involve an inventive step, and are capable of industrial application. The comment further suggested a response. *Response:* TRIPs Article 27 does not address what must be included in a patent application to allow WTO Member officials to determine whether particular inventions meet the standards for patentability established in that Article. TRIPs Article 29, which is more relevant to this comment, states that Members "shall require" patent applicants to disclose their invention "in a manner sufficiently clear and complete for the invention to be carried out by a person skilled in the art." If the written description is not clear and complete, the applicant may not have been in possession of the invention. This may support both written description and enablement standards. In addition, Article 29 expressly authorizes Members to require patent applicants to disclose the best method the inventor knows at the time of filing an application for carrying out the invention.

(29) *Comment:* Two comments commended the USPTO for eliminating the Biotechnology Specific Examples in the Revised Interim Written Description Guidelines and providing separate training materials. One comment indicated a need to reconfirm the examples set forth in the Interim Written Description Guidelines published in 1998. *Response:* The current training materials reflect the manner in which the USPTO interprets the Written Description Guidelines.

(30) *Comment:* Several comments addressed specific concerns about the examiner training materials. *Response:* The comments received with respect to the training materials will be taken under advisement as the Office revises the training materials in view of the revisions to the Guidelines. The specific comments will not be addressed herein as they do not impact the language of the Guidelines.

Guidelines for the Examination of Patent Applications Under the 35 U.S.C. 112, ¶ 1, "Written Description" Requirement

These "Written Description Guidelines" are intended to assist Office personnel in the examination of patent applications for compliance with the written description requirement of 35 U.S.C. 112, ¶ 1. This revision is based on the Office's current understanding of the law and public comments received in response to the USPTO's previous request for public comments on its Revised Interim Written Description Guidelines and is believed to be fully consistent with binding precedent of the U.S. Supreme Court, as well as the U.S. Court of Appeals for the Federal Circuit and its predecessor courts.

This revision does not constitute substantive rulemaking and hence does not have the force and effect of law. It is designed to assist Office personnel in analyzing claimed subject matter for compliance with substantive law. Rejections will be based upon the substantive law, and it is these rejections which are appealable. Consequently, any perceived failure by Office personnel to follow these Guidelines is neither appealable nor petitionable.

These Guidelines are intended to form part of the normal examination process. Thus, where Office personnel establish a *prima facie* case of lack of written description for a claim, a thorough review of the prior art and examination on the merits for compliance with the other statutory requirements, including those of 35 U.S.C. 101, 102, 103, and 112, is to be conducted prior to completing an Office action which includes a rejection for lack of written description. Office personnel are to rely on this revision of the Guidelines in the event of any inconsistent treatment of issues involving the written description requirement between these Guidelines and any earlier guidance provided from the Office.

I. General Principles Governing Compliance With the "Written Description" Requirement for Applications

The first paragraph of 35 U.S.C. 112 requires that the "specification shall contain a written description of the invention * * *." This requirement is separate and distinct from the enablement requirement.[1] The written description requirement has several policy objectives. "[T]he 'essential goal' of the description of the invention requirement is to clearly convey the information that an applicant has invented the subject matter which is claimed."[2] Another objective is to put the public in possession of what the applicant claims as the invention.[3] The written description requirement of the Patent Act promotes the progress of the useful arts by ensuring that patentees adequately describe their inventions in their patent specifications in exchange for the right to exclude others from practicing the invention for the duration of the patent's term.

To satisfy the written description requirement, a patent specification must describe the claimed invention in sufficient detail that one skilled in the art can reasonably conclude that the inventor had possession of the claimed invention.[4] An applicant shows possession of the claimed invention by describing the claimed invention with all of its limitations using such descriptive means as words, structures, figures, diagrams, and formulas that fully set forth the claimed invention.[5] Possession may be shown in a variety of ways including description of an actual reduction to practice,[6] or by showing that the invention was "ready for patenting" such as by the disclosure of drawings or structural chemical formulas that show that the invention was complete,[7] or by describing distinguishing identifying characteristics sufficient to show that the applicant was in possession of the claimed invention.[8] A question as to whether a specification provides an adequate written description may arise in the context of an original claim which is not described sufficiently, a new or amended claim wherein a claim limitation has been added or removed, or a claim to entitlement of an earlier priority date or effective filing date under 35 U.S.C. 119, 120, or 365(c).[9] Compliance with the written description requirement is a question of

fact which must be resolved on a case-by-case basis.[10]

A. Original Claims

There is a strong presumption that an adequate written description of the claimed invention is present when the application is filed.[11] However, the issue of a lack of adequate written description may arise even for an original claim when an aspect of the claimed invention has not been described with sufficient particularity such that one skilled in the art would recognize that the applicant had possession of the claimed invention.[12] The claimed invention as a whole may not be adequately described if the claims require an essential or critical feature which is not adequately described in the specification and which is not conventional in the art or known to one of ordinary skill in the art.[13] This problem may arise where an invention is described solely in terms of a method of its making coupled with its function and there is no described or art-recognized correlation or relationship between the structure of the invention and its function.[14] A lack of adequate written description issue also arises if the knowledge and level of skill in the art would not permit one skilled in the art to immediately envisage the product claimed from the disclosed process.[15]

B. New or Amended Claims

The proscription against the introduction of new matter in a patent application[16] serves to prevent an applicant from adding information that goes beyond the subject matter originally filed.[17] Thus, the written description requirement prevents an applicant from claiming subject matter that was not adequately described in the specification as filed. New or amended claims which introduce elements or limitations which are not supported by the as-filed disclosure violate the written description requirement.[18] While there is no *in haec verba* requirement, newly added claim limitations must be supported in the specification through express, implicit, or inherent disclosure. An amendment to correct an obvious error does not constitute new matter where one skilled in the art would not only recognize the existence of the error in the specification, but also recognize the appropriate correction.[19] Deposits made after the application filing date cannot be relied upon to support additions to or correction of information in the application as filed.[20]

Under certain circumstances, omission of a limitation can raise an issue regarding whether the inventor had possession of a broader, more generic invention.[21] A claim that omits an element which applicant describes as an essential or critical feature of the invention originally disclosed does not comply with the written description requirement.[22]

The fundamental factual inquiry is whether the specification conveys with reasonable clarity to those skilled in the art that, as of the filing date sought, applicant was in possession of the invention as now claimed.[23]

II. Methodology for Determining Adequacy of Written Description

A. Read and Analyze the Specification for Compliance With 35 U.S.C. 112, ¶ 1

Office personnel should adhere to the following procedures when reviewing patent applications for compliance with the written description requirement of 35 U.S.C. 112, ¶ 1. The examiner has the initial burden, after a thorough reading and evaluation of the content of the application, of presenting evidence or reasons why a person skilled in the art would not recognize that the written description of the invention provides support for the claims. There is a strong presumption that an adequate written description of the claimed invention is present in the specification as filed;[24] however, with respect to newly added or amended claims, applicant should show support in the original disclosure for the new or amended claims.[25] Consequently, rejection of an original claim for lack of written description should be rare. The inquiry into whether the description requirement is met is a question of fact that must be determined on a case-by-case basis.[26]

1. For Each Claim, Determine What the Claim as a Whole Covers

Claim construction is an essential part of the examination process. Each claim must be separately analyzed and given its broadest reasonable interpretation in light of and consistent with the written description.[27] The entire claim must be considered, including the preamble language[28] and the transitional phrase.[29] The claim as a whole, including all limitations found in the preamble,[30] the transitional phrase, and the body of the claim, must be sufficiently supported to satisfy the written description requirement.[31]

The examiner should evaluate each claim to determine if sufficient structures, acts, or functions are recited to make clear the scope and meaning of the claim, including the weight to be given the preamble.[32] The absence of definitions or details for well-established terms or procedures should not be the basis of a rejection under 35 U.S.C. 112, ¶ 1, for lack of adequate written description. Limitations may not, however, be imported into the claims from the specification.

2. Review the Entire Application to Understand How Applicant Provides Support for the Claimed Invention Including Each Element and/or Step

Prior to determining whether the disclosure satisfies the written description requirement for the claimed subject matter, the examiner should review the claims and the entire specification, including the specific embodiments, figures, and sequence listings, to understand how applicant provides support for the various features of the claimed invention.[33] The analysis of whether the specification complies with the written description requirement calls for the examiner to compare the scope of the claim with the scope of the description to determine whether applicant has demonstrated possession of the claimed invention. Such a review is conducted from the standpoint of one of skill in the art at the time the application was filed[34] and should include a determination of the field of the invention and the level of skill and knowledge in the art. Generally, there is an inverse correlation between the level of skill and knowledge in the art and the specificity of disclosure necessary to satisfy the written description requirement. Information which is well known in the art need not be described in detail in the specification.[35]

3. Determine Whether There is Sufficient Written Description to Inform a Skilled Artisan That Applicant was in Possession of the Claimed Invention as a Whole at the Time the Application Was Filed

a. Original claims. Possession may be shown in many ways. For example, possession may be shown, *inter alia*, by describing an actual reduction to practice of the claimed invention. Possession may also be shown by a clear depiction of the invention in detailed drawings or in structural chemical formulas which permit a person skilled in the art to clearly recognize that applicant had possession of the claimed invention. An adequate written description of the invention may be shown by any description of sufficient, relevant, identifying characteristics so long as a person skilled in the art would recognize that the inventor had possession of the claimed invention.[36]

A specification may describe an actual reduction to practice by showing

that the inventor constructed an embodiment or performed a process that met all the limitations of the claim and determined that the invention would work for its intended purpose.[37] Description of an actual reduction to practice of a biological material may be shown by specifically describing a deposit made in accordance with the requirements of 37 CFR 1.801 *et seq.*[38]

An applicant may show possession of an invention by disclosure of drawings[39] or structural chemical formulas[40] that are sufficiently detailed to show that applicant was in possession of the claimed invention as a whole. The description need only describe in detail that which is new or not conventional.[41] This is equally true whether the claimed invention is directed to a product or a process.

An applicant may also show that an invention is complete by disclosure of sufficiently detailed, relevant identifying characteristics[42] which provide evidence that applicant was in possession of the claimed invention,[43] *i.e.*, complete or partial structure, other physical and/or chemical properties, functional characteristics when coupled with a known or disclosed correlation between function and structure, or some combination of such characteristics.[44] What is conventional or well known to one of ordinary skill in the art need not be disclosed in detail.[45] If a skilled artisan would have understood the inventor to be in possession of the claimed invention at the time of filing, even if every nuance of the claims is not explicitly described in the specification, then the adequate description requirement is met.[46]

(1) For each claim drawn to a single embodiment or species:[47]

(a) Determine whether the application describes an actual reduction to practice of the claimed invention.

(b) If the application does not describe an actual reduction to practice, determine whether the invention is complete as evidenced by a reduction to drawings or structural chemical formulas that are sufficiently detailed to show that applicant was in possession of the claimed invention as a whole.

(c) If the application does not describe an actual reduction to practice or reduction to drawings or structural chemical formula as discussed above, determine whether the invention has been set forth in terms of distinguishing identifying characteristics as evidenced by other descriptions of the invention that are sufficiently detailed to show that applicant was in possession of the claimed invention.

(i) Determine whether the application as filed describes the complete structure (or acts of a process) of the claimed invention as a whole. The complete structure of a species or embodiment typically satisfies the requirement that the description be set forth "in such full, clear, concise, and exact terms" to show possession of the claimed invention.[48] If a complete structure is disclosed, the written description requirement is satisfied for that species or embodiment, and a rejection under 35 U.S.C. 112, ¶ 1, for lack of written description must not be made.

(ii) If the application as filed does not disclose the complete structure (or acts of a process) of the claimed invention as a whole, determine whether the specification discloses other relevant identifying characteristics sufficient to describe the claimed invention in such full, clear, concise, and exact terms that a skilled artisan would recognize applicant was in possession of the claimed invention.[49]

Whether the specification shows that applicant was in possession of the claimed invention is not a single, simple determination, but rather is a factual determination reached by considering a number of factors. Factors to be considered in determining whether there is sufficient evidence of possession include the level of skill and knowledge in the art, partial structure, physical and/or chemical properties, functional characteristics alone or coupled with a known or disclosed correlation between structure and function, and the method of making the claimed invention. Disclosure of any combination of such identifying characteristics that distinguish the claimed invention from other materials and would lead one of skill in the art to the conclusion that the applicant was in possession of the claimed species is sufficient.[50] Patents and printed publications in the art should be relied upon to determine whether an art is mature and what the level of knowledge and skill is in the art. In most technologies which are mature, and wherein the knowledge and level of skill in the art is high, a written description question should not be raised for original claims even if the specification discloses only a method of making the invention and the function of the invention.[51] In contrast, for inventions in emerging and unpredictable technologies, or for inventions characterized by factors not reasonably predictable which are known to one of ordinary skill in the art, more evidence is required to show possession. For example, disclosure of only a method of making the invention and the function may not be sufficient to support a product claim other than a product-by-process claim.[52] Furthermore, disclosure of a partial structure without additional characterization of the product may not be sufficient to evidence possession of the claimed invention.[53]

Any claim to a species that does not meet the test described under at least one of (a), (b), or (c) must be rejected as lacking adequate written description under 35 U.S.C. 112, ¶ 1.

(2) For each claim drawn to a genus:

The written description requirement for a claimed genus may be satisfied through sufficient description of a representative number of species by actual reduction to practice (see (1)(a), above), reduction to drawings (see (1)(b), above), or by disclosure of relevant, identifying characteristics, *i.e.*, structure or other physical and/or chemical properties, by functional characteristics coupled with a known or disclosed correlation between function and structure, or by a combination of such identifying characteristics, sufficient to show the applicant was in possession of the claimed genus (see (1)(c), above).[54]

A "representative number of species" means that the species which are adequately described are representative of the entire genus. Thus, when there is substantial variation within the genus, one must describe a sufficient variety of species to reflect the variation within the genus. On the other hand, there may be situations where one species adequately supports a genus.[55] What constitutes a "representative number" is an inverse function of the skill and knowledge in the art. Satisfactory disclosure of a "representative number" depends on whether one of skill in the art would recognize that the applicant was in possession of the necessary common attributes or features of the elements possessed by the members of the genus in view of the species disclosed. For inventions in an unpredictable art, adequate written description of a genus which embraces widely variant species *cannot* be achieved by disclosing only one species within the genus.[56] Description of a representative number of species does not require the description to be of such specificity that it would provide individual support for each species that the genus embraces.[57] If a representative number of adequately described species are not disclosed for a genus, the claim to that genus must be rejected as lacking adequate written description under 35 U.S.C. 112, ¶ 1.

b. New claims, amended claims, or claims asserting entitlement to the benefit of an earlier priority date or filing date under 35 U.S.C. 119, 120, or

365(c). The examiner has the initial burden of presenting evidence or reasoning to explain why persons skilled in the art would not recognize in the original disclosure a description of the invention defined by the claims.[58] However, when filing an amendment an applicant should show support in the original disclosure for new or amended claims.[59] To comply with the written description requirement of 35 U.S.C. 112, ¶ 1, or to be entitled to an earlier priority date or filing date under 35 U.S.C. 119, 120, or 365(c), each claim limitation must be expressly,[60] implicitly,[61] or inherently[62] supported in the originally filed disclosure.[63] Furthermore, each claim must include all elements which applicant has described as essential.[64]

If the originally filed disclosure does not provide support for each claim limitation, or if an element which applicant describes as essential or critical is not claimed, a new or amended claim must be rejected under 35 U.S.C. 112, ¶ 1, as lacking adequate written description, or in the case of a claim for priority under 35 U.S.C. 119, 120, or 365(c), the claim for priority must be denied.

III. Complete Patentability Determination Under All Statutory Requirements and Clearly Communicate Findings, Conclusions, and Their Bases

The above only describes how to determine whether the written description requirement of 35 U.S.C. 112, ¶ 1, is satisfied. Regardless of the outcome of that determination, Office personnel must complete the patentability determination under all the relevant statutory provisions of title 35 of the U.S. Code.

Once Office personnel have concluded analysis of the claimed invention under all the statutory provisions, including 35 U.S.C. 101, 112, 102, and 103, they should review all the proposed rejections and their bases to confirm their correctness. Only then should any rejection be imposed in an Office action. The Office action should clearly communicate the findings, conclusions, and reasons which support them. When possible, the Office action should offer helpful suggestions on how to overcome rejections.

A. For Each Claim Lacking Written Description Support, Reject the Claim Under Section 112, ¶ 1, for Lack of Adequate Written Description

A description as filed is presumed to be adequate, unless or until sufficient evidence or reasoning to the contrary has been presented by the examiner to rebut the presumption.[65] The examiner, therefore, must have a reasonable basis to challenge the adequacy of the written description. The examiner has the initial burden of presenting by a preponderance of evidence why a person skilled in the art would not recognize in an applicant's disclosure a description of the invention defined by the claims.[66] In rejecting a claim, the examiner must set forth express findings of fact regarding the above analysis which support the lack of written description conclusion. These findings should:

(1) Identify the claim limitation at issue; and

(2) Establish a *prima facie* case by providing reasons why a person skilled in the art at the time the application was filed would not have recognized that the inventor was in possession of the invention as claimed in view of the disclosure of the application as filed. A general allegation of "unpredictability in the art" is not a sufficient reason to support a rejection for lack of adequate written description.

When appropriate, suggest amendments to the claims which can be supported by the application's written description, being mindful of the prohibition against the addition of new matter in the claims or description.[67]

B. Upon Reply by Applicant, Again Determine the Patentability of the Claimed Invention, Including Whether the Written Description Requirement Is Satisfied by Reperforming the Analysis Described Above in View of the Whole Record

Upon reply by applicant, before repeating any rejection under 35 U.S.C. 112, ¶ 1, for lack of written description, review the basis for the rejection in view of the record as a whole, including amendments, arguments, and any evidence submitted by applicant. If the whole record now demonstrates that the written description requirement is satisfied, do *not* repeat the rejection in the next Office action. If the record still does not demonstrate that the written description is adequate to support the claim(s), repeat the rejection under 35 U.S.C. 112, ¶ 1, fully respond to applicant's rebuttal arguments, and properly treat any further showings submitted by applicant in the reply. When a rejection is maintained, any affidavits relevant to the 112, ¶ 1, written description requirement,[68] must be thoroughly analyzed and discussed in the next Office action.

Dated: December 29, 2000.

Q. Todd Dickinson,

Under Secretary of Commerce for Intellectual Property and Director of the United States Patent and Trademark Office.

Endnotes

[1] *See, e.g., Vas-Cath, Inc.* v. *Mahurkar,* 935 F.2d 1555, 1560, 19 USPQ2d 1111, 1114 (Fed. Cir. 1991).

[2] *In re Barker,* 559 F.2d 588, 592 n.4, 194 USPQ 470, 473 n.4 (CCPA 1977).

[3] *See Regents of the University of California* v. *Eli Lilly,* 119 F.3d 1559, 1566, 43 USPQ2d 1398, 1404 (Fed. Cir. 1997), *cert. denied,* 523 U.S. 1089 (1998).

[4] *See, e.g., Vas-Cath, Inc.* v. *Mahurkar,* 935 F.2d at 1563, 19 USPQ2d at 1116. Much of the written description case law addresses whether the specification as originally filed supports claims not originally in the application. The issue raised in the cases is most often phrased as whether the original application provides "adequate support" for the claims at issue or whether the material added to the specification incorporates "new matter" in violation of 35 U.S.C. 132. The "written description" question similarly arises in the interference context, where the issue is whether the specification of one party to the interference can support the newly added claims corresponding to the count at issue, *i.e.,* whether that party can "make the claim" corresponding to the interference count. *See, e.g., Martin* v. *Mayer,* 823 F.2d 500, 503, 3 USPQ2d 1333, 1335 (Fed. Cir. 1987).

In addition, early opinions suggest the Patent and Trademark Office was unwilling to find written descriptive support when the only description was found in the claims; however, this viewpoint was rejected. *See In re Koller,* 613 F.2d 819, 204 USPQ 702 (CCPA 1980) (original claims constitute their own description); *accord In re Gardner,* 475 F.2d 1389, 177 USPQ 396 (CCPA 1973); *accord In re Wertheim,* 541 F.2d 257, 191 USPQ 90 (CCPA 1976) (accord). It is now well accepted that a satisfactory description may be in the claims or any other portion of the originally filed specification. These early opinions did not address the quality or specificity of particularity that was required in the description, *i.e.,* how much description is enough.

[5] *Lockwood* v. *American Airlines, Inc.,* 107 F.3d 1565, 1572, 41 USPQ2d 1961, 1966 (Fed. Cir. 1997).

[6] An application specification may show actual reduction to practice by describing testing of the claimed invention or, in the case of biological materials, by specifically describing a deposit made in accordance with 37 CFR 1.801 *et seq. See also Deposit of Biological Materials for Patent Purposes, Final Rule,* 54 FR 34,864 (August 22, 1989) ("The requirement for a specific identification is consistent with the description requirement of the first paragraph of 35 U.S.C. 112, and to provide an antecedent basis for the biological material which either has been or will be deposited before the patent is granted." *Id.* at 34,876. "The description must be sufficient to permit verification that the deposited biological material is in fact that disclosed. Once the

patent issues, the description must be sufficient to aid in the resolution of questions of infringement." *Id.* at 34,880.). Such a deposit is not a substitute for a written description of the claimed invention. The written description of the deposited material needs to be as complete as possible because the examination for patentability proceeds solely on the basis of the written description. *See, e.g., In re Lundak*, 773 F.2d 1216, 227 USPQ 90 (Fed. Cir. 1985). *See also* 54 FR at 34,880 ("As a general rule, the more information that is provided about a particular deposited biological material, the better the examiner will be able to compare the identity and characteristics of the deposited biological material with the prior art.").

[7] *Pfaff* v. *Wells Electronics, Inc.*, 525 U.S. 55, 68, 119 S.Ct. 304, 312, 48 USPQ2d 1641, 1647 (1998); *Eli Lilly*, 119 F.3d at 1568, 43 USPQ2d at 1406.

[8] *See Amgen, Inc.* v. *Chugai Pharmaceutical*, 927 F.2d 1200, 1206, 18 USPQ2d 1016, 1021 (Fed. Cir. 1991) (one must define a compound by "whatever characteristics sufficiently distinguish it").

[9] A description requirement issue can arise for original claims (*see, e.g., Eli Lilly*, 119 F.3d 1559, 43 USPQ2d 1398) as well as new or amended claims. Most typically, the issue will arise in the context of determining whether new or amended claims are supported by the description of the invention in the application as filed (*see, e.g., In re Wright*, 866 F.2d 422, 9 USPQ2d 1649 (Fed. Cir. 1989)), whether a claimed invention is entitled to the benefit of an earlier priority date or effective filing date under 35 U.S.C. 119, 120, or 365(c) (*see, e.g., Tronzo* v. *Biomet, Inc.*, 156 F.3d 1154, 47 USPQ2d 1829 (Fed. Cir. 1998); *Fiers* v. *Revel*, 984 F.2d 1164, 25 USPQ2d 1601 (Fed. Cir. 1993); *In re Ziegler*, 992 F.2d 1197, 1200, 26 USPQ2d 1600, 1603 (Fed. Cir. 1993)), or whether a specification provides support for a claim corresponding to a count in an interference (*see, e.g., Fields* v. *Conover*, 443 F.2d 1386, 170 USPQ 276 (CCPA 1971)).

[10] *Vas-Cath, Inc.* v. *Mahurkar*, 935 F.2d at 1563, 19 USPQ2d at 1116 (Fed. Cir. 1991).

[11] *In re Wertheim*, 541 F.2d 257, 263, 191 USPQ 90, 97 (CCPA 1976) ("we are of the opinion that the PTO has the initial burden of presenting evidence or reasons why persons skilled in the art would not recognize in the disclosure a description of the invention defined by the claims").

[12] See endnote 4.

[13] For example, consider the claim "A gene comprising SEQ ID NO:1." A determination of what the claim as a whole covers may result in a conclusion that specific structures such as a promoter, a coding region, or other elements are included. Although all genes encompassed by this claim share the characteristic of comprising SEQ ID NO:1, there may be insufficient description of those specific structures (*e.g.*, promoters, enhancers, coding regions, and other regulatory elements) which are also included.

[14] A biomolecule sequence described only by a functional characteristic, without any known or disclosed correlation between that function and the structure of the sequence, normally is not a sufficient identifying characteristic for written description purposes, even when accompanied by a method of obtaining the claimed sequence. For example, even though a genetic code table would correlate a known amino acid sequence with a genus of coding nucleic acids, the same table cannot predict the native, naturally occurring nucleic acid sequence of a naturally occurring mRNA or its corresponding cDNA. *Cf. In re Bell*, 991 F.2d 781, 26 USPQ2d 1529 (Fed. Cir. 1993), and *In re Deuel*, 51 F.3d 1552, 34 USPQ2d 1210 (Fed. Cir. 1995) (holding that a process could not render the product of that process obvious under 35 U.S.C. 103). The Federal Circuit has pointed out that under United States law, a description that does not render a claimed invention obvious cannot sufficiently describe the invention for the purposes of the written description requirement of 35 U.S.C. 112. *Eli Lilly*, 119 F.3d at 1567, 43 USPQ2d at 1405.

Compare Fonar Corp. v. *General Electric Co.*, 107 F.3d 1543, 1549, 41 USPQ2d 1801, 1805 (Fed. Cir. 1997) ("As a general rule, where software constitutes part of a best mode of carrying out an invention, description of such a best mode is satisfied by a disclosure of the functions of the software. This is because, normally, writing code for such software is within the skill of the art, not requiring undue experimentation, once its functions have been disclosed. * * * Thus, flow charts or source code listings are not a requirement for adequately disclosing the functions of software.").

[15] *See, e.g., Fujikawa* v. *Wattanasin*, 93 F.3d 1559, 1571, 39 USPQ2d 1895, 1905 (Fed. Cir. 1996) (a "laundry list" disclosure of every possible moiety does not constitute a written description of every species in a genus because it would not "reasonably lead" those skilled in the art to any particular species); *In re Ruschig*, 379 F.2d 990, 995, 154 USPQ 118, 123 (CCPA 1967) ("*If* n-propylamine had been used in making the compound instead of n-butylamine, the compound of claim 13 would have resulted. Appellants submit to us, as they did to the board, an imaginary specific example patterned on specific example 6 by which the above butyl compound is made so that we can see what a simple change would have resulted in a specific supporting disclosure being present in the present specification. The trouble is that there is no such disclosure, easy though it is to imagine it.") (emphasis in original); *Purdue Pharma L.P.* v. *Faulding Inc.*, 230 F.3d 1320, 1328, 56 USPQ2d 1481, 1487 (Fed. Cir. 2000) ("the specification does not clearly disclose to the skilled artisan that the inventors * * * considered the [] ratio to be part of their invention * * *. There is therefore no force to Purdue's argument that the written description requirement was satisfied because the disclosure revealed a broad invention from which the [later-filed] claims carved out a patentable portion").

[16] 35 U.S.C. §§ 132 and 251. *See also In re Rasmussen*, 650 F.2d 1212, 1214, 211 USPQ 323, 326 (CCPA 1981). *See* Manual of Patent Examining Procedure (MPEP) §§ 2163.06–2163.07 (7th Ed., Rev. 1, Feb. 2000) for a more detailed discussion of the written description requirement and its relationship to new matter.

[17] The claims as filed in the original specification are part of the disclosure and, therefore, if an application as originally filed contains a claim disclosing material not found in the remainder of the specification, the applicant may amend the specification to include the claimed subject matter. *In re Benno*, 768 F.2d 1340, 226 USPQ 683 (Fed. Cir. 1985).

[18] *See, e.g., In re Lukach*, 442 F.2d 967, 169 USPQ 795 (CCPA 1971) (subgenus range was not supported by generic disclosure and specific example within the subgenus range); *In re Smith*, 458 F.2d 1389, 1395, 173 USPQ 679, 683 (CCPA 1972) (a subgenus is not necessarily described by a genus encompassing it and a species upon which it reads).

[19] *In re Oda*, 443 F.2d 1200, 170 USPQ 260 (CCPA 1971). With respect to the correction of sequencing errors in applications disclosing nucleic acid and/or amino acid sequences, it is well known that sequencing errors are a common problem in molecular biology. *See, e.g.*, Peter Richterich, *Estimation of Errors in 'Raw' DNA Sequences: A Validation Study*, 8 Genome Research 251–59 (1998). If an application as filed includes sequence information and references a deposit of the sequenced material made in accordance with the requirements of 37 CFR § 1.801 *et seq.*, amendment may be permissible.

[20] Corrections of minor errors in the sequence may be possible based on the argument that one of skill in the art would have resequenced the deposited material and would have immediately recognized the minor error. Deposits made after the filing date can only be relied upon to provide support for the correction of sequence information if applicant submits a statement in compliance with 37 CFR § 1.804 stating that the biological material which is deposited is a biological material specifically defined in the application as filed.

[21] *See, e.g., Gentry Gallery, Inc.* v. *Berkline Corp.*, 134 F.3d 1473, 45 USPQ2d 1498 (Fed. Cir. 1998) (claims to a sectional sofa comprising, *inter alia*, a console and a control means were held invalid for failing to satisfy the written description requirement where the claims were broadened by removing the location of the control means.); *Johnson Worldwide Associates* v. *Zebco Corp.*, 175 F.3d 985, 993, 50 USPQ2d 1607, 1613 (Fed. Cir. 1999) (In *Gentry Gallery*, the "court's determination that the patent disclosure did not support a broad meaning for the disputed claim terms was premised on clear statements in the written description that described the location of a claim element—the 'control means'—as 'the only possible location' and that variations were 'outside the stated purpose of the invention.' *Gentry Gallery*, 134 F.3d at 1479, 45 USPQ2d at 1503. *Gentry Gallery*, then, considers the situation where the patent's disclosure makes crystal clear that a particular (*i.e.*, narrow) understanding of a claim term is an 'essential element of [the inventor's] invention.' "); *Tronzo* v. *Biomet*, 156 F.3d at 1158–59, 47 USPQ2d at 1833 (Fed. Cir. 1998) (claims to generic cup shape were not entitled to filing date of parent application which disclosed "conical cup" in view of the disclosure of the

parent application stating the advantages and importance of the conical shape.).

[22] *See Gentry Gallery*, 134 F.3d at 1480, 45 USPQ2d at 1503; *In re Sus*, 306 F.2d 494, 504, 134 USPQ 301, 309 (CCPA 1962) ("[O]ne skilled in this art would not be taught by the written description of the invention in the specification that any 'aryl or substituted aryl radical' would be suitable for the purposes of the invention but rather that only *certain aryl radicals* and certain specifically substituted aryl radicals [*i.e.*, aryl azides] would be suitable for such purposes.") (emphasis in original). A claim which omits matter disclosed to be essential to the invention as described in the specification or in other statements of record may also be subject to rejection under 35 U.S.C. 112, ¶ 1, as not enabling, or under 35 U.S.C. 112, ¶ 2. *See In re Mayhew*, 527 F.2d 1229, 188 USPQ 356 (CCPA 1976); *In re Venezia*, 530 F.2d 956, 189 USPQ 149 (CCPA 1976); and *In re Collier*, 397 F.2d 1003, 158 USPQ 266 (CCPA 1968). *See also* MPEP § 2172.01.

[23] *See, e.g., Vas-Cath, Inc.*, 935 F.2d at 1563–64, 19 USPQ2d at 1117.

[24] *Wertheim*, 541 F.2d at 262, 191 USPQ at 96.

[25] *See* MPEP §§ 714.02 and 2163.06 ("Applicant should * * * specifically point out the support for any amendments made to the disclosure."); and MPEP § 2163.04 ("If applicant amends the claims and points out where and/or how the originally filed disclosure supports the amendment(s), and the examiner finds that the disclosure does not reasonably convey that the inventor had possession of the subject matter of the amendment at the time of the filing of the application, the examiner has the initial burden of presenting evidence or reasoning to explain why persons skilled in the art would not recognize in the disclosure a description of the invention defined by the claims.").

[26] *See In re Smith*, 458 F.2d 1389, 1395, 173 USPQ 679, 683 (CCPA 1972) ("Precisely how close [to the claimed invention] the description must come to comply with § 112 must be left to case-by-case development."); *In re Wertheim*, 541 F.2d at 262, 191 USPQ at 96 (inquiry is primarily factual and depends on the nature of the invention and the amount of knowledge imparted to those skilled in the art by the disclosure).

[27] *See, e.g., In re Morris*, 127 F.3d 1048, 1053–54, 44 USPQ2d 1023, 1027 (Fed. Cir. 1997).

[28] "Preamble language" is that language in a claim appearing before the transitional phase, *e.g.*, before "comprising," "consisting essentially of," or "consisting of."

[29] The transitional term "comprising" (and other comparable terms, *e.g.*, "containing," "including," and "having") is "open-ended—it covers the expressly recited subject matter, alone or in combination with unrecited subject matter. *See, e.g., Genentech, Inc.* v. *Chiron Corp.*, 112 F.3d 495, 501, 42 USPQ2d 1608, 1613 (Fed. Cir. 1997) (" 'Comprising' is a term of art used in claim language which means that the named elements are essential, but other elements may be added and still form a construct within the scope of the claim."); *Ex parte Davis*, 80 USPQ 448, 450 (Bd. App. 1948) ("comprising" leaves the "claim open for the inclusion of unspecified ingredients even in major amounts"). "By using the term 'consisting essentially of,' the drafter signals that the invention necessarily includes the listed ingredients and is open to unlisted ingredients that do not materially affect the basic and novel properties of the invention. A 'consisting essentially of' claim occupies a middle ground between closed claims that are written in a 'consisting of' format and fully open claims that are drafted in a 'comprising' format." *PPG Industries* v. *Guardian Industries*, 156 F.3d 1351, 1354, 48 USPQ2d 1351, 1353–54 (Fed. Cir. 1998). For the purposes of searching for and applying prior art under 35 U.S.C. 102 and 103, absent a clear indication in the specification or claims of what the basic and novel characteristics actually are, 'consisting essentially of' will be construed as equivalent to "comprising." *See, e.g., PPG*, 156 F.3d at 1355, 48 USPQ2d at 1355 ("PPG could have defined the scope of the phrase "consisting essentially of" for purposes of its patent by making clear in its specification what it regarded as constituting a material change in the basic and novel characteristics of the invention."). *See also In re Janakirama-Rao*, 317 F.2d 951, 954, 137 USPQ 893, 895–96 (CCPA 1963). If an applicant contends that additional steps or materials in the prior art are excluded by the recitation of "consisting essentially of," applicant has the burden of showing that the introduction of additional steps or components would materially change the characteristics of applicant's invention. *In re De Lajarte*, 337 F.2d 870, 143 USPQ 256 (CCPA 1964).

[30] *See Pac-Tec Inc.* v. *Amerace Corp.*, 903 F.2d 796, 801, 14 USPQ2d 1871, 1876 (Fed. Cir. 1990) (determining that preamble language that constitutes a structural limitation is actually part of the claimed invention).

[31] An applicant shows possession of the claimed invention by describing the claimed invention with all of its limitations. *Lockwood*, 107 F.3d at 1572, 41 USPQ2d at 1966.

[32] *See, e.g., Bell Communications Research, Inc.* v. *Vitalink Communications Corp.*, 55 F.3d 615, 620, 34 USPQ2d 1816, 1820 (Fed. Cir. 1995) ("[A] claim preamble has the import that the claim as a whole suggests for it."); *Corning Glass Works* v. *Sumitomo Elec. U.S.A., Inc.*, 868 F.2d 1251, 1257, 9 USPQ2d 1962, 1966 (Fed. Cir. 1989) (The determination of whether preamble recitations are structural limitations can be resolved only on review of the entirety of the application "to gain an understanding of what the inventors actually invented and intended to encompass by the claim.").

[33] An element may be critical where those of skill in the art would require it to determine that applicant was in possession of the invention. *Compare Rasmussen*, 650 F.2d at 1215, 211 USPQ at 327 ("one skilled in the art who read Rasmussen's specification would understand that it is unimportant *how* the layers are adhered, so long as they are adhered") (emphasis in original), *with Amgen, Inc.* v. *Chugai Pharmaceutical Co., Ltd.*, 927 F.2d 1200, 1206, 18 USPQ2d 1016, 1021 (Fed. Cir. 1991) ("it is well established in our law that conception of a chemical compound requires that the inventor be able to define it so as to distinguish it from other materials, and to describe how to obtain it").

[34] *See, e.g., Wang Labs.* v. *Toshiba Corp.*, 993 F.2d 858, 865, 26 USPQ2d 1767, 1774 (Fed. Cir. 1993).

[35] *See, e.g., Hybritech, Inc.* v. *Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 1379–80, 231 USPQ 81, 90 (Fed. Cir. 1986).

[36] *See, e.g., Purdue Pharma L.P.* v. *Faulding Inc.*, 230 F.3d 1320, ___, 56 USPQ2d 1481, 1483 (Fed. Cir. 2000) (the written description "inquiry is a factual one and must be assessed on a case-by-case basis"); *see also Pfaff* v. *Wells Electronics, Inc.*, 55 U.S. at 66, 119 S.Ct. at 311, 48 USPQ2d at 1646 ("The word 'invention' must refer to a concept that is complete, rather than merely one that is 'substantially complete.' It is true that reduction to practice ordinarily provides the best evidence that an invention is complete. But just because reduction to practice is sufficient evidence of completion, it does not follow that proof of reduction to practice is necessary in every case. Indeed, both the facts of the *Telephone Cases* and the facts of this case demonstrate that one can prove that an invention is complete and ready for patenting before it has actually been reduced to practice.").

[37] *Cooper* v. *Goldfarb*, 154 F.3d 1321, 1327, 47 USPQ2d 1896, 1901 (Fed. Cir. 1998). *See also UMC Elecs. Co.* v. *United States*, 816 F.2d 647, 652, 2 USPQ2d 1465, 1468 (Fed. Cir. 1987) ("[T]here cannot be a reduction to practice of the invention * * * without a physical embodiment which includes all limitations of the claim."); *Estee Lauder Inc.* v. *L'Oreal, S.A.*, 129 F.3d 588, 593, 44 USPQ2d 1610, 1614 (Fed. Cir. 1997) ("[A] reduction to practice does not occur until the inventor has determined that the invention will work for its intended purpose."); *Mahurkar* v. *C.R. Bard, Inc.*, 79 F.3d 1572, 1578, 38 USPQ2d 1288, 1291 (Fed. Cir. 1996) (determining that the invention will work for its intended purpose may require testing depending on the character of the invention and the problem it solves).

[38] 37 CFR 1.804, 1.809. *See also* endnote 6.

[39] *See, e.g., Vas-Cath*, 935 F.2d at 1565, 19 USPQ2d at 1118 ("drawings alone may provide a 'written description' of an invention as required by § 112"); *In re Wolfensperger*, 302 F.2d 950, 133 USPQ 537 (CCPA 1962) (the drawings of applicant's specification provided sufficient written descriptive support for the claim limitation at issue); *Autogiro Co. of America* v. *United States*, 384 F.2d 391, 398, 155 USPQ 697, 703 (Ct. Cl. 1967) ("In those instances where a visual representation can flesh out words, drawings may be used in the same manner and with the same limitations as the specification.").

[40] *See, e.g., Eli Lilly*, 119 F.3d at 1568, 43 USPQ2d at 1406 ("In claims involving chemical materials, generic formulae usually indicate with specificity what the generic claims encompass. One skilled in the art can distinguish such a formula from others and can identify many of the species that the claims encompass. Accordingly, such a formula is normally an adequate description of the claimed genus.").

[41] *See Hybritech* v. *Monoclonal Antibodies*, 802 F.2d at 1384, 231 USPQ at 94; *Fonar Corp.* v. *General Electric Co.*, 107 F.3d at 1549, 41 USPQ2d at 1805 (source code description not required).

[42] For example, the presence of a restriction enzyme map of a gene may be relevant to a statement that the gene has been isolated. One skilled in the art may be able to determine when the gene disclosed is the same as or different from a gene isolated by another by comparing the restriction enzyme map. In contrast, evidence that the gene could be digested with a nuclease would not normally represent a relevant characteristic since any gene would be digested with a nuclease. Similarly, isolation of an mRNA and its expression to produce the protein of interest is strong evidence of possession of an mRNA for the protein.

For some biomolecules, examples of identifying characteristics include a sequence, structure, binding affinity, binding specificity, molecular weight, and length. Although structural formulas provide a convenient method of demonstrating possession of specific molecules, other identifying characteristics or combinations of characteristics may demonstrate the requisite possession. For example, unique cleavage by particular enzymes, isoelectric points of fragments, detailed restriction enzyme maps, a comparison of enzymatic activities, or antibody cross-reactivity may be sufficient to show possession of the claimed invention to one of skill in the art. *See Lockwood*, 107 F.3d at 1572, 41 USPQ2d at 1966 ("written description" requirement may be satisfied by using "such descriptive means as words, structures, figures, diagrams, formulas, etc., that fully set forth the claimed invention").

[43] A definition by function alone "does not suffice" to sufficiently describe a coding sequence "because it is only an indication of what the gene does, rather than what it is." *Eli Lilly*, 119 F.3 at 1568, 43 USPQ2d at 1406. *See also Fiers*, 984 F.2d at 1169–71, 25 USPQ2d at 1605–06 (discussing *Amgen Inc.* v. *Chugai Pharmaceutical Co.*, 927 F.2d 1200, 18 USPQ2d 1016 (Fed. Cir. 1991)).

[44] If a claim limitation invokes 35 U.S.C. 112, ¶ 6, it must be interpreted to cover the corresponding structure, materials, or acts in the specification and "equivalents thereof." *See* 35 U.S.C. 112, ¶ 6. *See also B. Braun Medical, Inc.* v. *Abbott Lab.*, 124 F.3d 1419, 1424, 43 USPQ2d 1896, 1899 (Fed. Cir. 1997). In considering whether there is 35 U.S.C. 112, ¶ 1, support for a means- (or step) plus-function claim limitation, the examiner must consider not only the original disclosure contained in the summary and detailed description of the invention portions of the specification, but also the original claims, abstract, and drawings. A means- (or step-) plus-function claim limitation is adequately described under 35 U.S.C. 112, ¶ 1, if: (1) The written description adequately links or associates adequately described particular structure, material, or acts to the function recited in a means- (or step-) plus-function claim limitation; or (2) it is clear based on the facts of the application that one skilled in the art would have known what structure, material, or acts perform the function recited in a means- (or step-) plus-function limitation. Note also: A rejection under 35 U.S.C. 112, ¶ 2, "cannot stand where there is adequate description in the specification to satisfy 35 U.S.C. 112, first paragraph, regarding means-plus-function recitations that are not, per se, challenged for being unclear." *In re Noll*, 545 F.2d 141, 149, 191 USPQ 721, 727 (CCPA 1976). *See Supplemental Examination Guidelines for Determining the Applicability of 35 U.S.C. 112, ¶ 6*, 65 FR 38510, June 21, 2000.

[45] *See Hybritech Inc.* v. *Monoclonal Antibodies, Inc.*, 802 F.2d at 1384, 231 USPQ at 94.

[46] *See, e.g., Vas-Cath*, 935 F.2d at 1563, 19 USPQ2d at 1116; *Martin* v. *Johnson*, 454 F.2d 746, 751, 172 USPQ 391, 395 (CCPA 1972) (stating "the description need not be in *ipsis verbis* [*i.e.*, "in the same words"] to be sufficient").

[47] A claim which is limited to a single disclosed embodiment or species is analyzed as a claim drawn to a single embodiment or species, whereas a claim which encompasses two or more embodiments or species within the scope of the claim is analyzed as a claim drawn to a genus. *See also* MPEP § 806.04(e).

[48] 35 U.S.C. 112, ¶ 1. *Cf. Fields* v. *Conover*, 443 F.2d 1386, 1392, 170 USPQ 276, 280 (CCPA 1971) (finding a lack of written description because the specification lacked the "full, clear, concise, and exact written description" which is necessary to support the claimed invention).

[49] For example, if the art has established a strong correlation between structure and function, one skilled in the art would be able to predict with a reasonable degree of confidence the structure of the claimed invention from a recitation of its function. Thus, the written description requirement may be satisfied through disclosure of function and minimal structure when there is a well-established correlation between structure and function. In contrast, without such a correlation, the capability to recognize or understand the structure from the mere recitation of function and minimal structure is highly unlikely. In this latter case, disclosure of function alone is little more than a wish for possession; it does not satisfy the written description requirement. *See Eli Lilly*, 119 F.3d at 1568, 43 USPQ2d at 1406 (written description requirement not satisfied by merely providing "a result that one might achieve if one made that invention"); *In re Wilder*, 736 F.2d 1516, 1521, 222 USPQ 369, 372–73 (Fed. Cir. 1984) (affirming a rejection for lack of written description because the specification does "little more than outline goals appellants hope the claimed invention achieves and the problems the invention will hopefully ameliorate"). *Compare Fonar*, 107 F.3d at 1549, 41 USPQ2d at 1805 (disclosure of software function adequate in that art).

[50] *See Eli Lilly*, 119 F.3d at 1568, 43 USPQ2d at 1406.

[51] *See, e.g., In re Hayes Microcomputer Products, Inc. Patent Litigation*, 982 F.2d 1527, 1534–35, 25 USPQ2d 1241, 1246 (Fed. Cir. 1992) ("One skilled in the art would know how to program a microprocessor to perform the necessary steps described in the specification. Thus, an inventor is not required to describe every detail of his invention. An applicant's disclosure obligation varies according to the art to which the invention pertains. Disclosing a microprocessor capable of performing certain functions is sufficient to satisfy the requirement of section 112, first paragraph, when one skilled in the relevant art would understand what is intended and know how to carry it out.")

[52] *See, e.g., Fiers* v. *Revel*, 984 F.2d at 1169, 25 USPQ2d at 1605; *Amgen.*, 927 F.2d at 1206, 18 USPQ2d at1021. Where the process has actually been used to produce the product, the written description requirement for a product-by-process claim is clearly satisfied; however, the requirement may not be satisfied where it is not clear that the acts set forth in the specification can be performed, or that the product is produced by that process.

[53] *See, e.g., Amgen*, 927 F.2d at1206, 18 USPQ2d at 1021 ("A gene is a chemical compound, albeit a complex one, and it is well established in our law that conception of a chemical compound requires that the inventor be able to define it so as to distinguish it from other materials, and to describe how to obtain it. Conception does not occur unless one has a mental picture of the structure of the chemical, or is able to define it by its method of preparation, its physical or chemical properties, or whatever characteristics sufficiently distinguish it. It is not sufficient to define it solely by its principal biological property, *e.g.*, encoding human erythropoietin, because an alleged conception having no more specificity than that is simply a wish to know the identity of any material with that biological property. We hold that when an inventor is unable to envision the detailed constitution of a gene so as to distinguish it from other materials, as well as a method for obtaining it, conception has not been achieved until reduction to practice has occurred, *i.e.*, until after the gene has been isolated.") (citations omitted). In such instances the alleged conception fails not merely because the field is unpredictable or because of the general uncertainty surrounding experimental sciences, but because the conception is incomplete due to factual uncertainty that undermines the specificity of the inventor's idea of the invention. *Burroughs Wellcome Co.* v. *Barr Laboratories Inc.*, 40 F.3d 1223, 1229, 32 USPQ2d 1915, 1920 (Fed. Cir. 1994). Reduction to practice in effect provides the only evidence to corroborate conception (and therefore possession) of the invention. *Id.*

[54] *See Eli Lilly*, 119 F.3d at 1568, 43 USPQ2d at 1406.

[55] *See, e.g., Rasmussen*, 650 F.2d at 1214, 211 USPQ at 326–27 (disclosure of a single method of adheringly applying one layer to another was sufficient to support a generic claim to "adheringly applying" because one skilled in the art reading the specification would understand that it is unimportant how the layers are adhered, so long as they are adhered); *In re Herschler*, 591 F.2d 693, 697, 200 USPQ 711, 714 (CCPA 1979) (disclosure of corticosteriod in DMSO sufficient to support claims drawn to a method of using a mixture of a "physiologically active steroid" and DMSO because "use of known chemical compounds in a manner auxiliary

to the invention must have a corresponding written description only so specific as to lead one having ordinary skill in the art to that class of compounds. Occasionally, a functional recitation of those known compounds in the specification may be sufficient as that description."); *In re Smythe*, 480 F.2d 1376, 1383, 178 USPQ 279, 285 (CCPA 1973) (the phrase "air or other gas which is inert to the liquid" was sufficient to support a claim to "inert fluid media" because the description of the properties and functions of the air or other gas segmentizing medium would suggest to a person skilled in the art that appellant's invention includes the use of "inert fluid" broadly.). However, in *Tronzo* v. *Biomet*, 156 F.3d at 1159, 47 USPQ2d at1833 (Fed. Cir. 1998), the disclosure of a species in the parent application did not suffice to provide written description support for the genus in the child application.

[56] *See, e.g., Eli Lilly.*

[57] For example, in the molecular biology arts, if an applicant disclosed an amino acid sequence, it would be unnecessary to provide an explicit disclosure of nucleic acid sequences that encoded the amino acid sequence. Since the genetic code is widely known, a disclosure of an amino acid sequence would provide sufficient information such that one would accept that an applicant was in possession of the full genus of nucleic acids encoding a given amino acid sequence, but not necessarily any particular species. *Cf. In re Bell*, 991 F.2d 781, 785, 26 USPQ2d 1529, 1532 (Fed. Cir. 1993) and *In re Baird*, 16 F.3d 380, 382, 29 USPQ2d 1550, 1552 (Fed. Cir. 1994).

[58] *See Wertheim*, 541 F.2d at 263, 191 USPQ at 97 ("[T]he PTO has the initial burden of presenting evidence or reasons why persons skilled in the art would not recognize in the disclosure a description of the invention defined by the claims.").

[59] *See* MPEP §§ 714.02 and 2163.06 ("Applicant should * * * specifically point out the support for any amendments made to the disclosure.").

[60] *See, e.g., In re Wright*, 866 F.2d 422, 425, 9 USPQ2d 1649, 1651 (Fed. Cir. 1989) (Original specification for method of forming images using photosensitive microcapsules which describes removal of microcapsules from surface and warns that capsules not be disturbed prior to formation of image, unequivocally teaches absence of permanently fixed microcapsules and supports amended language of claims requiring that microcapsules be "not permanently fixed" to underlying surface, and therefore meets description requirement of 35 U.S.C. 112.).

[61] *See, e.g., In re Robins*, 429 F.2d 452, 456–57, 166 USPQ 552, 555 (CCPA 1970) ("[W]here no explicit description of a generic invention is to be found in the specification * * * mention of representative compounds may provide an implicit description upon which to base generic claim language."); *In re Smith*, 458 F.2d 1389, 1395, 173 USPQ 679, 683 (CCPA 1972) (a subgenus is not necessarily implicitly described by a genus encompassing it and a species upon which it reads).

[62] *See, e.g., In re Robertson*, 169 F.3d 743, 745, 49 USPQ2d 1949, 1950–51 (Fed. Cir. 1999) ("To establish inherency, the extrinsic evidence "must make clear that the missing descriptive matter is necessarily present in the thing described in the reference, and that it would be so recognized by persons of ordinary skill. Inherency, however, may not be established by probabilities or possibilities. The mere fact that a certain thing may result from a given set of circumstances is not sufficient.'") (citations omitted).

[63] When an explicit limitation in a claim "is not present in the written description whose benefit is sought it must be shown that a person of ordinary skill would have understood, at the time the patent application was filed, that the description requires that limitation." *Hyatt* v. *Boone*, 146 F.3d 1348, 1353, 47 USPQ2d 1128, 1131 (Fed. Cir. 1998).

[64] *See, e.g., Johnson Worldwide Associates Inc.* v. *Zebco Corp.*, 175 F.3d at 993, 50 USPQ2d at 1613; *Gentry Gallery, Inc.* v. *Berkline Corp.*, 134 F.3d at 1479, 45 USPQ2d at 1503; *Tronzo* v. *Biomet*, 156 F.3d at 1159, 47 USPQ2d at 1833.

[65] *See, e.g., In re Marzocchi*, 439 F.2d 220, 224, 169 USPQ 367, 370 (CCPA 1971).

[66] *Wertheim*, 541 F.2d at 263, 191 USPQ at 97.

[67] *See Rasmussen*, 650 F.2d at 1214, 211 USPQ at 326.

[68] *See In re Alton*, 76 F.3d 1168, 1176, 37 USPQ2d 1578, 1584 (Fed. Cir. 1996).

[FR Doc. 01–323 Filed 1–4–01; 8:45 am]
BILLING CODE 3510–16–U

CORPORATION FOR NATIONAL AND COMMUNITY SERVICE

Revision of Currently Approved Information Collection; Comment Request

AGENCY: Corporation for National and Community Service

ACTION: Notice.

SUMMARY: The Corporation for National and Community Service (hereinafter "Corporation"), as part of its continuing effort to reduce paperwork and respondent burden, conducts a preclearance consultation program to provide the general public and Federal agencies with an opportunity to comment on proposed and/or continuing collections of information in accordance with the Paperwork Reduction Act of 1995 (PRA95) (44 U.S.C. 3506(c)(2)(A)). This program helps to ensure that requested data can be provided in the desired format, reporting burden (time and financial resources) is minimized, collection instruments are clearly understood, and the impact of collection requirement on respondents can be properly assessed.

Currently, the Corporation is soliciting comments concerning the proposed revision of its Voucher and Payment Request Form (OMB #3045–0014).

Copies of the forms can be obtained by contacting the office listed below in the address section of this notice.

DATES: Written comments must be submitted to the office listed in the **ADDRESSES** section by March 6, 2001.

ADDRESSES: Send comments to Levon Buller, National Service Trust, Corporation for National and Community Service, 1201 New York Ave., NW., Washington, DC 20525.

FOR FURTHER INFORMATION CONTACT: Levon Buller, (202) 606–5000, ext. 383.

SUPPLEMENTARY INFORMATION: The Corporation is particularly interested in comments which:

- Evaluate whether the proposed collection of information is necessary for the proper performance of the functions of the Corporation, including whether the information will have practical utility;
- Evaluate the accuracy of the agency's estimate of the burden of the proposed collection of information, including the validity of the methodology and assumptions used;
- Enhance the quality, utility and clarity of the information to be collected; and
- Minimize the burden of the collection of information on those who are to respond, including through the use of appropriate automated, electronic, mechanical, or other technological collection techniques or other forms of information technology, *e.g.*, permitting electronic submissions of responses.

Background

The Corporation supports programs that provide opportunities for individuals who want to become involved in national service. The service opportunities cover a wide range of activities over varying periods of time. Upon successfully completing an agreed-upon term of service in an approved AmeriCorps program, a national service participant—an AmeriCorps member—receives an "education award". This award is an amount of money set aside in the member's name in the National Service Trust Fund. This education award can be used to make payments towards qualified student loan or pay for educational expenses at qualified post-secondary institutions and approved school-to-work opportunities programs. Members have seven years in which to draw against any unused balance.

The National Service Trust is the office within the Corporation that administers the education award

Ref 5

ANNALS OF THE NEW YORK ACADEMY OF SCIENCES

Volume 556

HEPARIN AND RELATED POLYSACCHARIDES

STRUCTURE AND ACTIVITIES

Edited by Frederick A. Ofosu, Isidore Danishefsky, and Jack Hirsh

The New York Academy of Sciences
New York, New York
1989

Heparin, Heparan Sulfate, Smooth Muscle Cells, and Atherosclerosis[a]

M. J. KARNOVSKY,[b] T. C. WRIGHT, JR.,[b]
J. J. CASTELLOT, JR.,[b] J. CHOAY,[c] J.-C. LORMEAU,[c]
AND M. PETITOU[c]

[b]*Department of Pathology*
Harvard Medical School
Boston, Massachusetts

[c]*Institut Choay*
75782 Paris-Cedex 16

INTRODUCTION

The proliferation of vascular smooth muscle cells (VSMC) is a frequent consequence following endothelial injury, and is, indeed, thought to be an important early pathogenetic event in the evolution of the atherosclerotic plaque. The pathophysiologic, cellular, and molecular mechanisms underlying VSMC proliferation in atherosclerosis have recently been reviewed by Ross.[1] Under normal circumstances, the responsiveness of cells to mitogenic stimuli appears to be stringently controlled.[2] In addition to mitogens and promoters of cell growth, a number of molecules have been described in recent years that inhibit the growth of various cell types. These include interferon, retinoic acid, and transforming growth factor-β (TGF-β). For the past decade or so, our laboratory has been studying the regulation of vascular cell growth. In particular, we pursued our original observation that the glycosaminoglycan, heparin, inhibits the growth VSMC both *in vivo*,[3,4] following endothelial injury,[5] and *in vitro*.[6] More recently, we have been investigating the mechanisms of action responsible for heparin's growth-inhibitory activity, and the specific structural requirements underlying this activity. Heparan sulfates, produced by endothelial cells (EC)[7] and VSMC,[8] are also growth inhibitory of VSMC. Production of VSMC-inhibitory heparan sulfates by VSMC *in vitro* suggests autocrine secretion and a degree of autoregulation of VSMC growth, reminiscent of the behavior of other growth inhibitors, such as TGF-β.[9] Similar to TGF-β is the fact that heparin/heparan can have bifunctional effects, *e.g.*, stimulatory for EC grown in the presence of fibroblast growth factor (FGF),[10] and inhibitory for VSMC.[6] Taken all in all, the data from our laboratory and that of others strongly suggest a role for heparin/heparan molecules in the growth regulation of VSMC. In this article, we will review our structure-function studies on the inhibitory effects of heparin on VSMC proliferation both *in vivo* and *in vitro*.

[a] This work was supported by National Institutes of Health grants HL 17747 and CA 14723 (MJK) and CA 39436 (TCW); JJC is an Established Investigator of the American Heart Association. Portions of this work were supported by the generous gifts of the Institut Choay, Paris, France.

IN VIVO STUDIES

In 1977, Clowes and Karnovsky[3] first showed that heparin inhibited the well-known proliferation of VSMC that occurs in arteries following endothelial injury. We developed an air-drying injury model for desiccating the endothelium of an

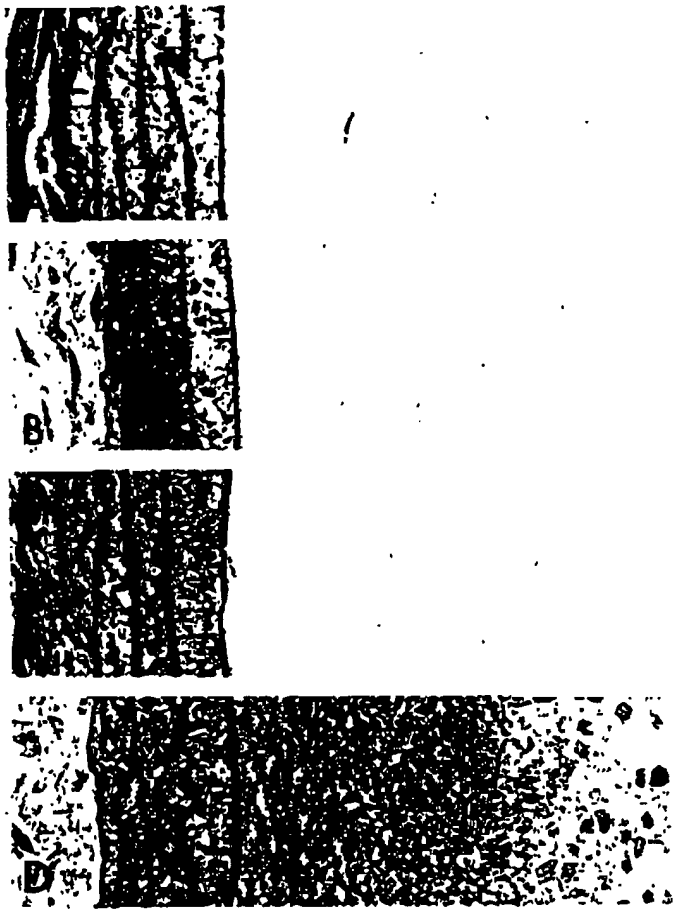


FIGURE 1. Composite histological figure illustrating the response of the rat carotid wall to endothelial denudation. To aid comparison of intimal thickening at each stage, the elastic laminae are aligned; the lumen is at the right in each panel, and the central area of the injured segment is shown in each case. The endothelium and thin intima are not visible at this magnification. (A) Normal, uninjured vessel. (B) Four days after denudation of endothelium there is edema of the media. (C) Seven days after denudation. (D) Fourteen days after injury, showing massive intimal thickening. (From Fishman *et al.*[5] Reproduced with permission from the U.S.-Canadian Academy of Pathology, Inc.)

isolated segment of the rat carotid;[5] upon restoration of blood flow, there was rapid deposition of platelets in the injured segment, followed within a few days by massive intimal proliferation of VSMC, presumably as a consequence of release of platelet-derived growth factor and other mitogens from the deposited platelets (FIG. 1). Similar results have been obtained by others using a balloon catheter

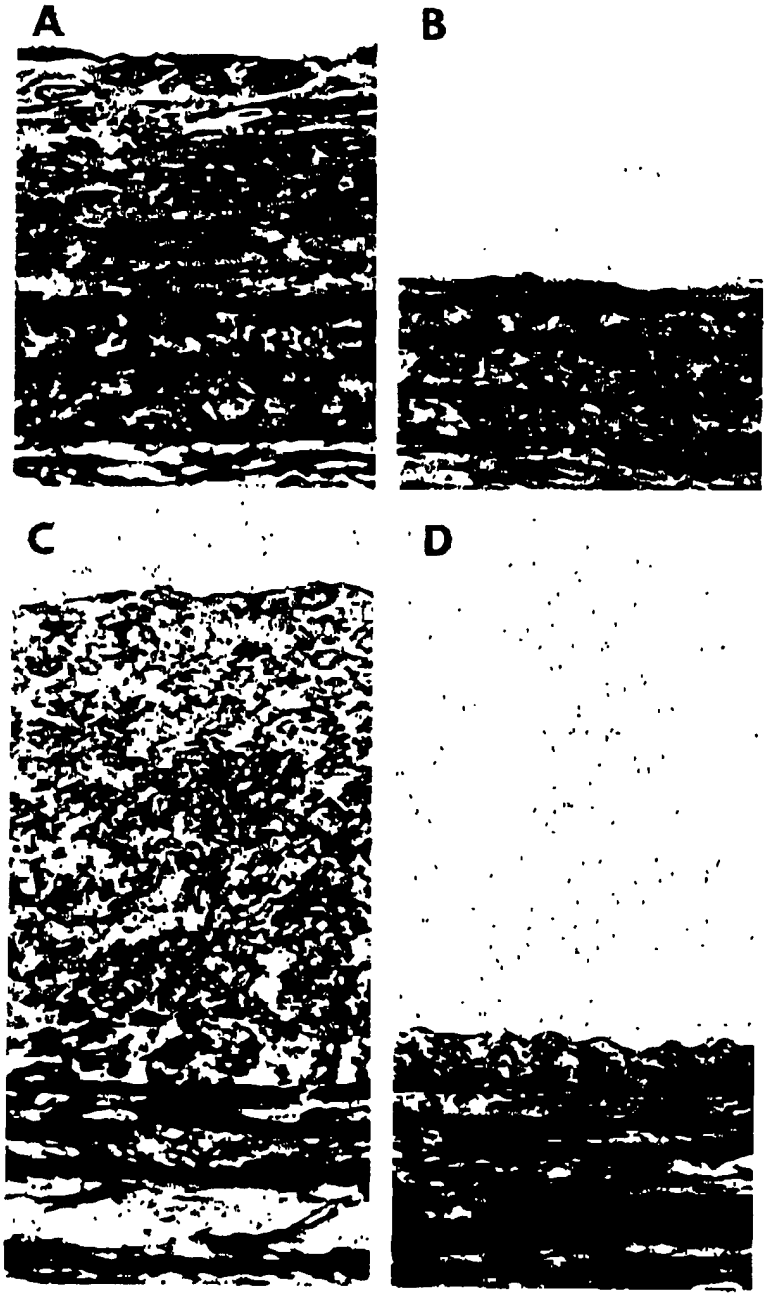


FIGURE 2. Appearance of intimal plaque near the midpoint of injured arterial segment fourteen days after injury. (A, C) Control rats not treated with heparin. Note the substantial myointimal proliferation in the control animals. (B) Rat given dose of anticoagulant heparin (0.5 mg/kg/h). Note the greatly reduced degree of myointimal thickening. (D) Rat treated with nonanticoagulant heparin (0.5 mg/kg/h). Note again the greatly reduced degree of myointimal thickening. (A and B from Clowes and Karnovsky,[3] Reproduced with permission from *Nature;* C and D from Guyton *et al.*,[4] Reproduced with permission from *Circulation Research.*)

injury model (*e.g.*, ref. 11). Interestingly, in our model, VSMC proliferation appeared to cease upon reendothelialization, leading us to hypothesize that the overlying layer of EC might in some fashion regulate VSMC proliferation.[5]

Because the clotting sequence is presumably activated in this vascular injury, and because thrombin had been shown to be a mitogen for some cell types,[12] we tested the effect of heparin in the rat carotid after the air-dry injury.[3] Heparin almost completely abolished VSMC hyperplasia following EC injury (FIG. 2); this effect was not due to changes in platelet functions or the rate of EC regeneration.[3]

The next question was whether this dramatic inhibitory effect was due to the anticoagulant (*i.e.*, antithrombin III-binding) activities of heparin, or was a separate nonanticoagulant activity. In collaboration with Dr. Robert Rosenberg, we tested heparin which had been separated into purified anticoagulant (AC) and nonanticoagulant (NAC) fractions, using antithrombin III affinity chromatography. Both fractions clearly inhibited VSMC proliferation in the injured carotid (FIG. 2), and, on a molar basis, both fractions were equally potent.[4] Therefore, antiproliferative activity is clearly separate from anticoagulant activity. Recently, Clowes and Clowes,[13] using a balloon catheter injury model in the rat carotid, have confirmed these observations, and showed, furthermore, that heparin blocked the migration of VSMC from the media to the intima as well. Similarly, in an experimental model for idiopathic pulmonary hypertension of the newborn, it was found that heparin inhibited the proliferation of VSMC in the pulmonary arteries of chronically hypoxic mice.[14]

In addition to these studies on VSMC, it should be noted that AC and NAC heparins also block the proliferation of mesangial cells, both *in vitro*[15] and in models of experimental proliferative glomerulonephritis *in vivo*.[16,17]

IN VITRO STUDIES

General

To develop a model system for analyzing the antiproliferative effect of heparin and derivatives on VSMC, we tested the ability of heparin to inhibit the growth of cultured VSMC. VSMC from a variety of vessels and species have been examined, including rat, calf and monkey aortae, rat mesenteric artery, and fetal bovine pulmonary arteries: all are markedly sensitive to the growth-inhibitory effects of heparin.[6,7] As pointed out above, mesangial cells are also very sensitive.[15] A number of studies on the effect of heparin on the proliferation of other cell types has been reported,[18,19] with contradictory results; we will not enter into a discussion of these observations. In our laboratory, when we tested heparin on a variety of cell types, we found that many cells, including EC, certain fibroblast cell lines, virally transformed cells, and Madin-Derby canine kidney epithelial cells were very much less sensitive than VSMC.[6,7] However, under specific conditions, rat cervical epithelial cells were sensitive;[20] this suggests that cells other than VSMC may be sensitive to heparin/heparan sulfate, and that the conditions required to reveal such sensitivity remain to be defined.

In this paper, we shall concentrate on studies involving VSMC. To study the growth effects of heparin *in vitro*, VSMC were growth arrested at subconfluent densities by serum deprivation to mimic more closely the *in vivo* situation, in which VSMC normally exist in a quiescent growth state.[21] The VSMC were released from the low-serum-induced growth block (*i.e.*, the G_0 state) by replacing the low-serum medium with normal growth medium containing 20% fetal calf

serum in the presence or absence of heparin or other test compounds[7] (FIG. 3). The ED_{50} for growth inhibition by heparin was 1 to 5 μg/ml, a level readily achieved in the blood of the animals in the *in vivo* models.

The growth-inhibitory effects of heparin *in vitro* are relatively specific for this glycosaminoglycan, and are unrelated to anticoagulant activities. Both AC and NAC heparins were highly effective as VSMC antiproliferative agents, whereas dermatan sulfate, chondroitin-4-sulfate and chondroitin-6-sulfate and hyaluronic acid were virtually ineffective[7] (FIG. 4). Lippman and Mathews[19] had previously shown that the antiproliferative effects of heparin on mouse L-M cells did not accord with anticoagulant activities. Our results with NAC extend those observations to the much more sensitive VSMC. It should be noted that heparin blocks VSMC growth late in G_1 and that the antiproliferative effects of AC and NAC are

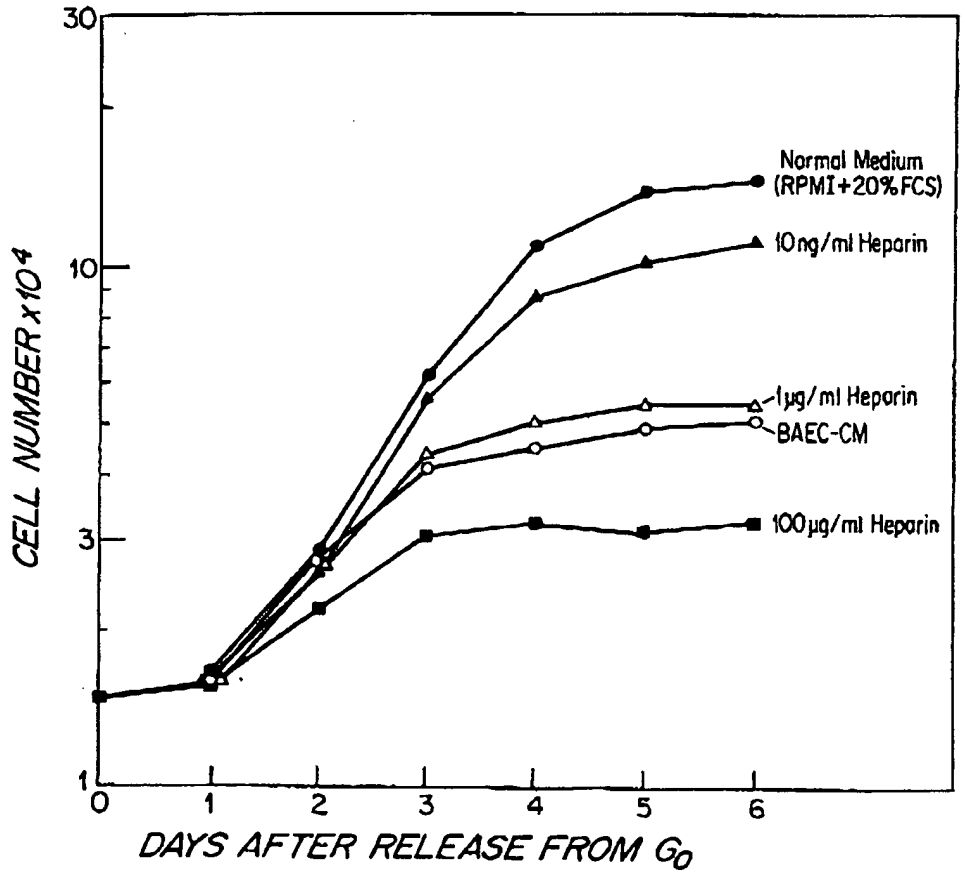


FIGURE 3. Effect of heparin and endothelial-cell-conditioned medium on VSMC growth. Sparsely plated rat aortic VSMC were growth-arrested by serum deprivation in G_0. Control cultures were released from the G_0 block by placing them in their normal growth medium (RPMI + 20% fetal calf serum). These cells proliferate until they are confluent. Other cultures were placed in a 1 : 1 mixture of bovine aortic endothelial-cell-conditioned medium (BAEC-CM) and RPMI + fetal calf serum (final serum concentration 20%), or in RPMI + 20% fetal calf serum containing the indicated concentrations of heparin. With increasing concentrations of heparin, there is progressive inhibition of VSMC growth. BAEC-CM also inhibits growth. Modified from Castellot *et al.*[7]

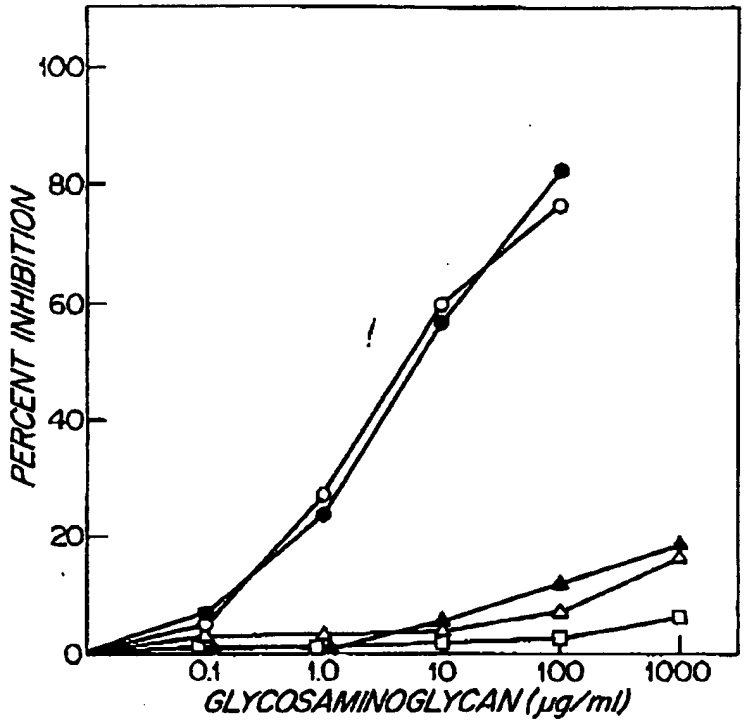


FIGURE 4. Effect of different glycosaminoglycans on rat SMC growth. (A) Cells were plated and growth-arrested for 48 hours in 0.4% serum. The cells were then exposed to fresh medium containing 20% FCS together with the indicated concentrations of glycosaminoglycans. Cell number was measured at daily intervals. Data are expressed as maximum percent inhibition. (B) Cells were plated at 6×10^3/16-mm dish. After 24 hours, the medium was aspirated and replated with fresh medium with 20% FCS with or without the indicated concentrations of glycosaminoglycans: ● = AC heparin, ○ = NAC heparin; ▲ = dermatan sulfate; △ = chrondroitin 4- and 6-sulfates; □ = hyaluronic acid. (From Castellot *et al.*[7])

fully reversible, *i.e.*, removal of heparin and replacement with normal medium allows the cells to enter S-phase and to resume their normal growth rate.[18]

To ascertain more precisely the molecular determinants of the antiproliferative effect of heparin for VSMC, we have undertaken structure-function studies, which will be reported on briefly below. Other cell biologic aspects of the inhibition of VSMC growth by heparin, such as effects on the cell cycle, growth factor-heparin interactions, binding and internalization of heparin, effects on VSMC metabolism, effects on the extracellular matrix, etc., will not be dealt with here, but have recently been reviewed in detail.[22,23]

STRUCTURE-FUNCTION STUDIES

Oligosaccharide Size Requirements for Antiproliferative Effect on VSMC

Two of us (MJK and JJC), initially in collaboration with Dr. Robert Rosenberg's laboratory, carried out experiments to establish what size of molecule of heparin is effective in inhibiting VSMC growth. Fragments of heparin were pre-

pared by limited digestion with nitrous acid, and the discrete oligosaccharide fragments that were generated were fractionated by gel filtration chromatography. The smallest heparin fragment that retained antiproliferative activity was a hexamer, whereas di- and tetrasaccharide fractions were inactive[24] (FIG. 5). The maximum antiproliferative activity resided in dodecamer and larger fragments.

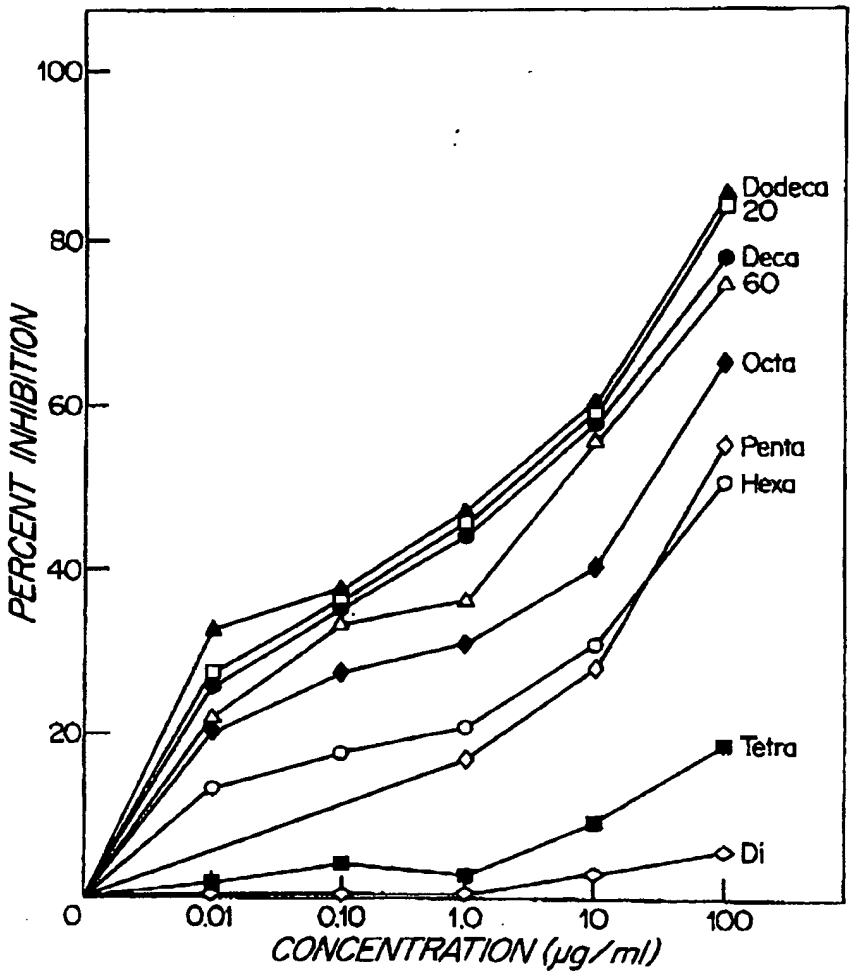


FIGURE 5. Effect of increasing concentrations of heparin fragments on growth of VSMC. Activity is expressed as percent inhibition of growth. The smallest effective molecules are the hexasaccharides derived from limited nitrous acid digestion of native heparin,[24] and the synthetic pentasaccharide.[25] (From Castellot *et al.*[24] Reproduced with permission of the *Journal of Cellular Physiology*.)

These latter activities were equivalent to native (*i.e.*, commercial) heparin at equimolecular doses.

Although the size fractions obtained by gel filtration are homogeneous with respect to size, they are quite heterogeneous with respect to their content of specific chemical structures. The use of synthetic heparin fragments overcomes this drawback.

These fragments, produced by Dr. Jean Choay and his colleagues,[25] are synthesized from defined monosaccharide units, and have an unambiguous structure. Using these well-defined oligosaccharides, we found that a pentasaccharide (having the unique structure for binding to antithrombin III) was found to have approximately the same antiproliferative activity as the hexasaccharide fraction obtained by nitrous acid digest of native heparin (FIG. 5). The synthetic tetrasaccharides, lacking either the reducing or non-reducing terminal sugar residue of the pentasaccharide, were only slightly antiproliferative.[26]

Influence of Sulfation on Antiproliferative Activity for VSMC

We (MJK and JJC) further examined, in collaboration with Dr. Robert Rosenberg's laboratory, the structural determinants of the antiproliferative effect of heparin on VSMC using chemically modified native (commercial) heparin.[24] Totally desulfated heparin lost all its anticoagulant and its antiproliferative activity; *N*-desulfated heparin lost most of its antiproliferative activity, as well as all its anticoagulant activity. Acetylation of the *N*-positions of the *N*-desulfated molecule restored most of the antiproliferative potency, but did not restore anticoagulant activity. *N*- and *O*-desulfated, *N*-resulfated molecules had no anticoagulant or inhibitory activity.

The above results indicate that *O*-sulfation—or at least a negative charge at the *O*-position—and a negative or neutral charge at the *N*-position, are required for antiproliferative activity; a positive charge at the *N*-position renders the molecules inactive.

The present authors have recently examined the relationship between size and charge in heparin-derived oligosaccharide fragments. The data is summarized below; a detailed report will be published elsewhere.[27] As found previously,[24] the native (unmodified) hexa- and octasaccharides were active, the latter more so, whereas the tetrasaccharide was inactive. However, *O*-oversulfation increased activity considerably for all fragments; for instance, the *O*-oversulfated tetrasaccharide became as active as the natural octasaccharide, suggesting that the degree of sulfation is an important factor in the antiproliferative activity (FIG. 6).

Interestingly, in contrast to native full-sized heparin (of approximately 40 saccharide residues)—which retain about 60% of the native activity upon *N*-desulfation, *N*-acetylation[24]—such modification of the fragments, including that of the octasaccharide, resulted in marked inactivation (FIG. 6). This result underscores the importance of size in relation to the antiproliferative activity. When the *N*-desulfated, *N*-acetylated oligosaccharides were *O*-oversulfated, they regained most of their native antiproliferative activities, *i.e.*, the hexa- and octasaccharides were once again active (FIG. 6). The degree of sulfation of the *N*-desulfated, *N*-acetylated, *O*-oversulfated fragments was similar to that of the unmodified native fragments, and the antiproliferative activities were similar for each pair of compounds. The importance of charge, especially at the *O*-position, is once again emphasized.

Although, in general, *O*-oversulfation enhanced activity of both native and modified fragments, when, within each group the relationship between size and charge was examined, it was clear that both the size of the oligosaccharides and the degree of sulfation were important in determining antiproliferative activity. For instance, in the case of *O*-oversulfation of native fragments, the ranking octa- > hexa- > tetrasaccharide was maintained, despite the increase in activity for each oligosaccharide over the unmodified molecule (FIG. 6). Similar relationships

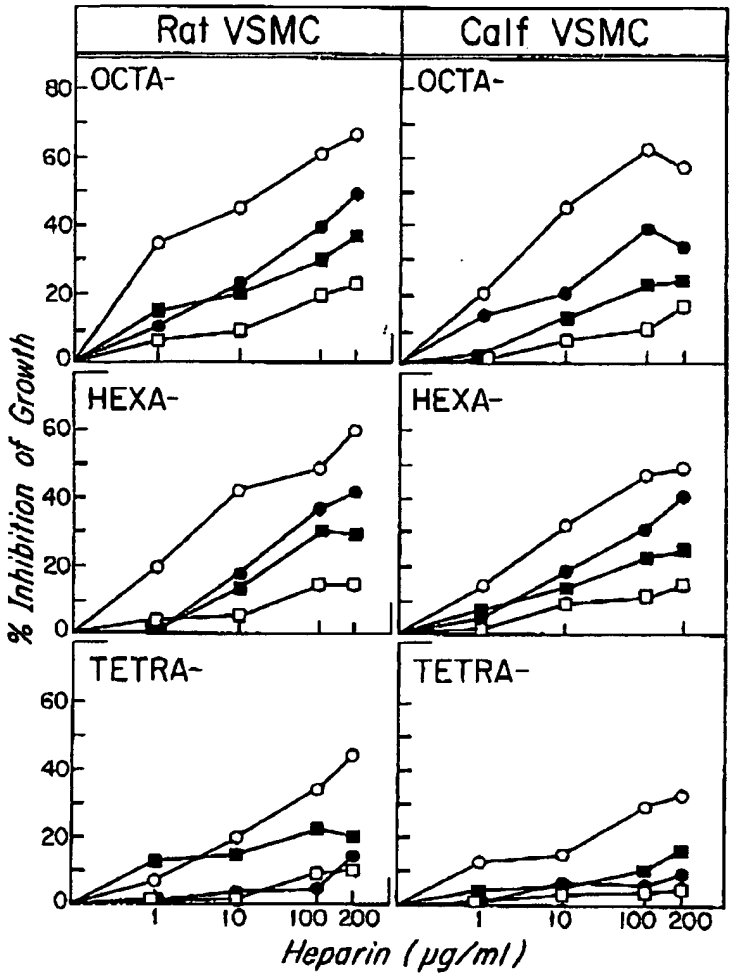


FIGURE 6. Effect of chemical modification and charge on antiproliferative activity of oligosaccharides. Cell number was determined after treatment for 5 to 7 days with the various concentrations of chemically modified octa-, hexa- and tetrasaccharides. Both rat VSMC and calf VSMC were released from G_0 block on day 0. Growth inhibition is expressed as percent inhibition of growth: ○ = oversulfated oligosaccharides; ● = unmodified oligosaccharides; ■ = *N*-desulfated, *N*-acetylated, *O*-oversulfated; □ = *N*-desulfated, *N*-acetylated. (From Wright *et al.*[27])

are mentioned above for the *N*-desulfated, *N*-acetylated, *O*-oversulfated oligosaccharides.

Another approach to analyzing the interdependence of size and charge on antiproliferative activity was to use hexasaccharides prepared from native heparin by nitrous acid cleavage, with subsequent separation into fractions of differing size densities on the basis of ion-exchange chromatography. These preparations allowed for testing of a wide range of naturally occurring charge densities within a given size of oligosaccharide. With hexasaccharide fragments, there was clearly increasing growth inhibition as the charge of the oligosaccharide increased, and the most charged hexasaccharide fraction had equivalent potency to the nonfractionated oligosaccharide[27] (FIG. 7).

Role of Antithrombin-III-binding Sequence and 3-O-Sulfate Glucosamine

As mentioned above, the synthetic pentasaccharide that is identical to the sequence required for heparin-antithrombin III binding[25] has antiproliferative activity as well. Removal of the 3-*O*-sulfate on the internal glucosamine almost completely abolished the antiproliferative activity. The importance of the other sulfates is currently being tested. As we have clearly shown that NAC heparin (*i.e.*, heparin that does not bind to antithrombin III) is also antiproliferative,[4,6,7] it is, at first sight, somewhat paradoxical that the pentasaccharide sequence unique for binding to antithrombin III[25] is antiproliferative as well,[26] and that both the binding and the antiproliferative activity are dependent on the 3-*O*-sulfate on the internal glucosamine.[25,26] This suggests that the sequence is but one of several antiproliferative sequences or structures. Furthermore, the 3-*O*-sulfate group on the internal glucosamine residue cannot explain the antiproliferative activity completely, since the tetrasaccharides lacking saccharides at either the reducing or nonreducing ends of the pentasaccharide contain the 3-*O*-sulfate, but are relatively inactive.[26]

To test for the overall importance of the unique pentasaccharide sequence, the present authors tested antiproliferative heparin preparations that do not have the pentasaccharide sequence and therefore lack the ability to bind to antithrombin III.[27] The pentasaccharide sequence contains an unsulfated glucuronic acid, and

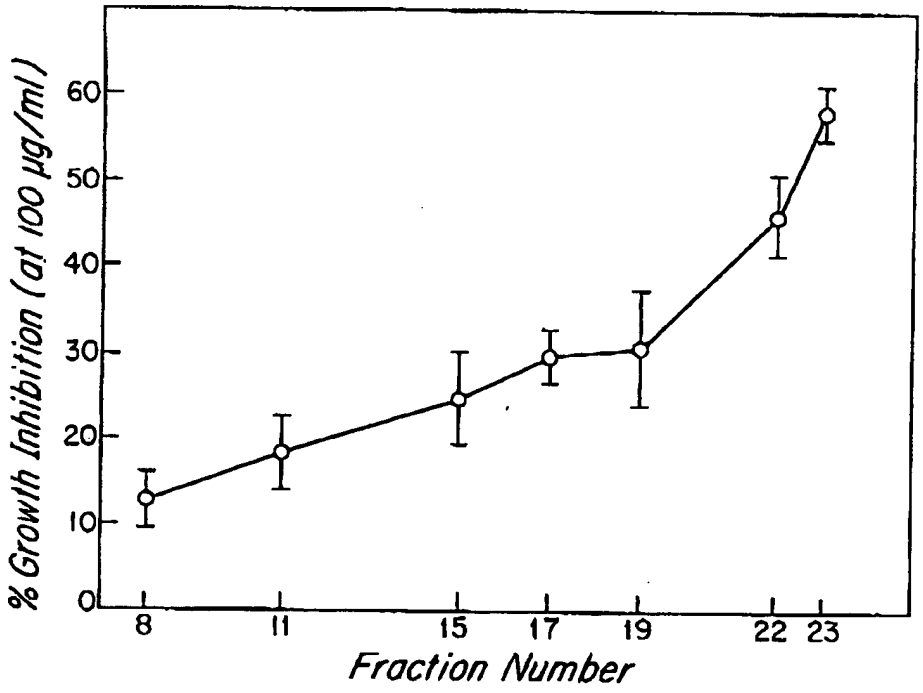


FIGURE 7. Antiproliferative activity of hexasaccharide fractions separated on the basis of charge. Hexasaccharides prepared from native heparin were separated into fractions of varying charge by ion-exchange chromatography. The greater the fraction number, the greater the negative charge. The percentage growth inhibition caused by 100 µg/ml of each of the fractions was determined after 5 to 7 days' exposure of rat VSMC released from G_0 block on day 0. (From Wright *et al.*[27])

therefore this moiety would be lost during depolymerization of heparin with periodic acid. We found that two heparin preparations treated with periodic acid, both of which lacked glucuronic acid residues as shown by ^{13}C-NMR, had significant antiproliferative activity. This indicates that the pentasaccharide antithrombin-III-binding sequence is not required for antiproliferative activity.

An important concomitant of the studies with the pure, synthetic heparin fragments is that it is highly unlikely that the growth inhibitory effect that we and others observe with native preparations is due to a nonheparin contaminant in these heterogeneous mixtures.

Role of 2-O-Sulfate Glucuronic Acid

Recently, Conrad and coworkers have demonstrated that heparan sulfate is present in the culture medium, the pericellular matrix, cytoplasm, and nucleus of cultured rat hepatocytes.[28,29] The rate of synthesis, the location, and the structure of the heparan sulfate species present were dependent on the growth state of the cells. They made the intriguing observation that the nuclear pool of heparan sulfate from confluent, nonproliferating cells, contained an unusually high proportion of 2-*O*-sulfated glucuronate residues, suggesting the interesting possibility that 2-*O*-sulfated glucuronic-acid-containing sequences are important in growth regulation. This led us to investigate whether or not the presence of 2-*O*-sulfate glucuronic acid is important for antiproliferative activity. For this purpose, two compounds lacking 2-*O*-sulfate glucuronic acid residues were prepared by Dr. Choay's group by controlled periodic acid depolymerization of native heparin. Both had significant antiproliferative activity for VSMC, low binding to antithrombin III, and totally lacked a ^{13}C-NMR signal corresponding to 2-*O*-sulfate residues. Similarly, the antiproliferative activity of fragments prepared by controlled periodic acid and controlled nitrous acid depolymerization were compared. The former contained low levels of 2-*O*-sulfate glucuronic acid levels as compared with the latter, but both had significant antiproliferative activity.

The above data show that 2-*O*-sulfate glucuronic acid residues are not necessary for antiproliferative activity. These observations do not, of course, gainsay the possibility that heparan sulfates containing 2-*O*-sulfate glucuronic acid residues do play an important antiproliferative role in growth regulation *in vivo*.

Dissociation between Antiproliferative and Anticoagulant Properties of Heparin and Heparin Fragments

It is now clear from this and previous data[4,6,7,26] that the antiproliferative and anticoagulant properties of heparin are quite separate. The NAC heparin prepared by antithrombin-III-affinity chromatography is highly antiproliferative but does not bind to antithrombin III and thus has extremely low thrombin-inhibitory activity.[30] Furthermore, since the ability of heparin to inhibit factor Xa and kallikrein depends upon the heparin-antithrombin-III-binding interaction,[31,32] the antiproliferative and anti-Xa and antikallikrein activities are likewise dissociated. We have found that a high molecular weight heparin fragment obtained by periodate oxidation of native heparin was likewise highly antiproliferative, but possessed little of the anti-IIa and anti-Xa activities of native heparin.[27] Contrariwise, the synthetic pentasaccharide species unique for binding to antithrombin III has 20-

fold more anti-Xa activity than native heparin on a molar basis, but only 10% to 20% of the antiproliferative activity.

The data indicate that the antiproliferative effect of heparin is independent of its ability to bind to antithrombin III, and is not mediated by interactions with serine proteases of the coagulation cascade such as thrombin (factor IIa), factor Xa, and kallikrein. As the structural determinants of the antiproliferative activity are very similar to those required for the ability of heparin to inhibit the serine protease human C3 convertase,[33] the possibility exists that the antiproliferative activity of heparin is exerted through its inhibitory effects on serine proteases other than IIa, Xa, and kallikrein. This possibility remains to be explored.

Structure-Function Relationship and Possible Clinical Applications

VSMC proliferation frequently occurs as a consequence of vascular injury. In addition to being an early event in the pathogenesis of atherosclerosis, VSMC proliferation apparently occurs not infrequently, and with considerable morbidity, following vascular surgery.[34] In forms of pulmonary hypertension such as idiopathic pulmonary hypertension of the newborn, VSMC proliferation is also an important pathognomic feature.

Thus, in addition to the intrinsic biologic interest of characterizing the antiproliferative features of heparin in structural-functional terms, there is considerable interest in utilizing heparin or heparin-derived species, particularly in regard to preventing VSMC proliferation following vascular surgery. To these ends, NAC-type heparin derivatives and heparin analogues, of enhanced potency, might be desirable and useful. We and others have shown that AC[3] as well as NAC[4] heparins are efficacious in inhibiting VSMC proliferation in experimental animals, following endothelial injury. Studies are currently underway in our laboratory on the effects of AC and NAC on experimental vein grafts, and the accelerated atherosclerosis, characterized by massive VSMC proliferation, seen in heart transplants. In a chronic hypoxic model of pulmonary hypertension, it has been shown that AC and NAC heparin inhibit VSMC proliferation which is a feature of the lesion.[14] In a model of progressive focal glomerulosclerosis, we were able to blunt, with both AC and NAC heparin, proteinuria, renal glomerular mesangial cell proliferation, and the progression of the disease in terms of glomerular functions and pathologic changes.[17] We ascribed these effects basically to the inhibition, by the heparins, of mesangial cell proliferation.[16-18]

In several areas, therefore, the potential in exploiting the antiproliferative effects of heparin and heparin-derived molecules in disease states seems worthy of further exploration.

REFERENCES

1. Ross, R. 1986. The pathogenesis of atherosclerosis—an update. N. Engl. J. Med. **314:** 488–500.
2. Sager, R. 1986. Genetic suppression of tumor formation: a new frontier in cancer research. Cancer Res. **46:** 1573–1580.
3. Clowes, A. W. & M. J. Karnovsky. 1977. Suppression by heparin of smooth muscle cell proliferation in injured arteries. Nature **265:** 625–626.
4. Guyton, J. R., R. D. Rosenberg, A. W. Clowes & M. J. Karnovsky. 1980. Inhibition of rat arterial smooth muscle cell proliferation by heparin. I. *In vivo* studies with anticoagulant and non-anticoagulant heparin. Circ. Res. **46:** 625–634.

5. FISHMAN, J. A., G. B. RYAN & M. J. KARNOVSKY. 1975. Endothelial regeneration in the rat carotid artery and the significance of endothelial denudation in the pathogenesis of myintimal thickening. Lab. Invest. 32: 339–351.
6. HOOVER, R. L., R. D. ROSENBERG, W. HAERING & M. J. KARNOVSKY. 1980. Inhibition of rat arterial smooth muscle cell proliferation by heparin. II. *In vitro* studies. Circ. Res. 47: 578–583.
7. CASTELLOT, J. J., JR., M. L. ADDONIZIO, R. D. ROSENBERG & M. J. KARNOVSKY. 1981. Cultured endothelial cells produce a heparin-like inhibitor of smooth muscle cell growth. J. Cell Biol. 90: 327–379.
8. FRITZE, L. M. S., C. F. REILLY & R. D. ROSENBERG. 1985. An antiproliferative heparan sulfate species produced by postconfluent smooth muscle cells. J. Cell Biol. 100: 1041–1049.
9. ROBERTS, A. B., M. A. ANZANO, L. M. WAKEFIELD, N. S. ROCHE, D. F. STERN & M. B. SPORN. 1985. Type beta transforming growth factor: a bifunctional regulator of cellular growth. Proc. Natl. Acad. Sci. U.S.A. 82: 119–123.
10. THORNTON, S. C., S. N. MUELLER & E. M. LEVINE. 1983. Human endothelial cells: use of heparin in cloning and long-term serial cultivation. Science 222: 623–625.
11. SCHWARTZ, S. M., C. HAUDENSCHILD & E. EDDY. 1978. Endothelial regeneration. I. Quantitative analysis of initial states of endothelial regeneration in rat aortic intima. Lab. Invest. 38: 568–580.
12. CHEN, L. B. & J. M. BUCHANAN. 1975. Mitogenic activity of blood components. I. Thrombin and prothrombin. Proc. Natl. Acad. Sci. U.S.A. 72: 131–135.
13. CLOWES, A. W. & M. M. CLOWES. 1985. Kinetics of cellular proliferation after arterial injury. II. Inhibition of smooth muscle growth by heparin. Lab. Invest. 52: 611–616.
14. HALES, C. A., R. L. KRADIN, R. D. BRANSTETTER & Y. ZHU. 1983. Impairment of hypoxic pulmonary artery remodeling by heparin in mice. Amer. Rev. Respir. Dis. 128: 747–751.
15. CASTELLOT, J. J., JR., R. L. HOOVER, P. A. HARPER & M. J. KARNOVSKY. 1985. Heparin and endothelial cell-secreted heparin-like species inhibit mesangial cell proliferation. Am. J. Pathol. 120: 427–435.
16. COFFEY, A. & M. J. KARNOVSKY. 1985. Heparin inhibits mesangial cell proliferation in Habu-venom-induced glomerular injury. Am. J. Pathol. 120: 248–255.
17. DIAMOND, J. R. & M. J. KARNOVSKY. 1986. Nonanticoagulant protective effect of heparin in chronic aminonucleoside nephrosis. Renal Physiol. Basel 3: 366–374.
18. CASTELLOT, J. J., JR., D. L. COCHRAN & M. J. KARNOVSKY. 1985. Effects of heparin on vascular smooth muscle cells. I. Cell metabolism. J. Cell. Physiol. 124: 21–28.
19. LIPPMAN, M. & M. B. MATHEWS. 1977. Heparins: varying effects on cell proliferation *in vitro* and lack of correlation with anticoagulant activity. Fed. Proc. 36: 55–59.
20. WRIGHT, T. C., JR., T. V. JOHNSTONE, J. J. CASTELLOT, JR., & M. J. KARNOVSKY. 1985. Inhibition of rat cervical epithelial cell growth by heparin and its reversal by EGF. J. Cell Physiol. 125: 499–506.
21. SCHWARTZ, S. M., C. M. GAJDUSEK & G. K. OWENS. 1982. Vessel growth control. *In* Pathobiology of the Endothelial Cell. H. L. Nossel & H. T. Vogt, Eds.: 63–78. Academic Press, New York, NY.
22. WRIGHT, T. C., JR., J. J. CASTELLOT, JR., J. R. DIAMOND & M. J. KARNOVSKY. 1988. Regulation of cellular proliferation by heparin and heparan sulfate. *In* Heparin. D. A. Lane & U. Lindahl, Eds. Edward Arnold, London, England. In press.
23. CASTELLOT, J. J., JR., T. C. WRIGHT, JR., & M. J. KARNOVSKY. 1987. Regulation of vascular smooth muscle cell growth by heparin. *In* Seminars in Thrombosis and Hemostasis. D. M. Stern & P. P. Nawroth, Eds. Vol. 13: 489–503. Thieme, Inc., New York, NY.
24. CASTELLOT, J. J., JR., D. L. BEELER, R. D. ROSENBERG & M. J. KARNOVSKY. 1984. Structural determinants of the capacity of heparin to inhibit the proliferation of vascular smooth muscle cells. J. Cell Physiol. 120: 315–320.
25. CHOAY, J., M. PETITOU, J.-C. LORMEAU, P. SINAY, B. CASU & G. GATTI. 1983. Structure-activity relationship in heparin: a synthetic pentasaccharide with high affinity for antithrombin III and eliciting high anti-factor Xa activity. Biochem. Biophys. Res. Commun. 116: 492–499.

26. CASTELLOT, J. J., JR., J. CHOAY, J.-C. LORMEAU, M. PETITOU, E. SACHE & M. J. KARNOVSKY. 1986. Structural determinants of the capacity of heparin to inhibit the proliferation of vascular smooth muscle cells. II. Evidence for a pentasaccharide sequence containing a 3-O-sulfate group. J. Cell Biol. **102**: 1979–1984.
27. WRIGHT, T. C., JR., J. J. CASTELLOT, JR., J. CHOAY, J.-C. LORMEAU, M. PETITOU & M. J. KARNOVSKY. 1989. Structural determinants of the growth inhibitory activity of heparin: interdependency of oligosaccharide size and charge. J. Biol. Chem. In press.
28. ISHIHARA, M., N. S. FEDARKO & H. E. CONRAD. 1986. Transport of heparan sulfate in the nuclei of hepatocytes. J. Biol. Chem. **261**: 13575–13580.
29. FEDARKO, N. S. & H. E. CONRAD. 1986. A unique heparan sulfate in the nuclei of hepatocytes: structural changes with the growth state of the cells. J. Cell Biol. **102**: 587–599.
30. LAM, L. H., J. E. SILBERT & R. D. ROSENBERG. 1976. The separation of active and inactive forms of heparin. Biochem. Biophys. Res. Commun. **69**: 570–577.
31. BURROWES, C. E., F. M. HABEL & H. Z. MOVAT. 1975. The inhibition of human plasma kallikrein by antithrombin III. Thromb. Res. **7**: 175–183.
32. VENNEROD, A. M. & K. LAAKE. 1975. Inhibition of purified plasma kallikrein by antithrombin III and heparin. Thromb. Res. **7**: 223–226.
33. KAZATCHKINE, M. D., D. T. FEARON, D. D. METCALFE, R. D. ROSENBERG & K. F. AUSTEN. 1981. Structural determinants of the capacity of heparin to inhibit formation of the human amplification of C3 convertase. J. Clin. Invest. **67**: 223–228.
34. WHITTEMORE, A. D., A. W. CLOWES, N. P. COUGH & J. A. MANNICK. 1981. Secondary femoropopliteal reconstruction. Ann. Surg. **193**: 35.

Biochem. J. (1994) 302, 625–639 (Printed in Great Britain)

REVIEW ARTICLE

The biology of perlecan: the multifaceted heparan sulphate proteoglycan of basement membranes and pericellular matrices

Renato V. IOZZO,* Isabelle R. COHEN, Susanne GRÄSSEL and Alan D. MURDOCH

Department of Pathology and Cell Biology and the Jefferson Cancer Institute, Thomas Jefferson University, Room 249, Jefferson Alumni Hall, 1020 Locust Street, Philadelphia, PA 19107, U.S.A.

INTRODUCTION

In the pre-molecular era of biochemistry, scientists assigned rather unprepossessing physiological roles to proteoglycans: they merely provided an inert scaffolding upon which cellular and organismal development could take place. The preceding decade, however, has witnessed an explosion of innovative technology which has created an interest in, and brought to the fore, a wealth of information in the field of proteoglycan research. It has originated a whole new perspective in our thinking of proteoglycan gene structure, function and evolution. We are entering the golden age of proteoglycan research and are on the brink of discovering human genetic diseases linked to specific proteoglycan gene families.

Perlecan is a multifaceted, modular proteoglycan that is present in virtually all vascularized tissues with a distribution that is primarily, but not exclusively, confined to basement membranes. The complex pleiotropy of perlecan suggests that this gene product is involved in several developmental processes, at both the early and late stages of embryogenesis, as well as in pervasive human diseases such as cancer and diabetes.

This review provides a propitious opportunity to comment on some new horizons and research lines opened up principally by the recent molecular data on perlecan gene structure, expression and topology.

HISTORICAL AND GENERAL CONSIDERATIONS

The neologism perlecan, a mnemonic from *perl* (bead or gem-like) and *can* (glycosaminoglycan), was coined by Hassell and co-workers [1] to symbolize the 'beads-on-a-string' appearance of isolated perlecan molecules as visualized by rotary shadowing. This term is now widely accepted and designates a gene family encoding modular, relatively large and ubiquitous glycoproteins which, as we discuss below, may or may not carry covalently linked glycosaminoglycan side chains.

The story of perlecan began over two decades ago with three key observations: a role for proteoglycans in branching morphogenesis [2], the demonstration of spatially ordered polyanionic sites in the basal lamina of breast ducts [3], and the discovery of heparan sulphate in the glomerular basement membrane [4]. Perhaps the work that has had the greatest impact on our understanding of perlecan is that of Hassell and collaborators [5]. These investigators first purified in chemically useful amounts a large, low-density heparan sulphate proteoglycan from a mouse sarcoma that was known to synthesize excessive quantities of basement membrane constituents [6–8]. The subsequent availability of immunological reagents recognizing specific domains in the protein core, rather than in the heparan sulphate side chains, has stimulated research in other cellular systems. These landmark studies have undoubtedly stimulated the work on perlecan and have set the stage for more recent discoveries, including the molecular cloning of the cDNA, the elucidation of the protein-core modules, and the characterization and chromosomal mapping of the gene.

One of the truisms of recent research is the perception that the size of the perlecan protein core has gradually increased over time. From earlier two-digit estimates, it has now reached the three-digit range for the protein core (400–470 kDa) and over four-digit size for the fully glycosylated proteoglycan in certain cellular systems [9]. Molecular cloning and peptide sequence analyses have now definitively proven that perlecan is not only synthesized by Engelbreth–Holm–Swarm (EHS) tumour cells, but also by cells as disparate as melanoma [10], colon carcinoma [11], lung fibroblastic [12], endothelial [13], and mammary epithelial [14] cells. Perlecan is an archetypal molecule from which multiple species of proteoglycan could be derived either via a well-refined proteolytic processing pathway or via a pattern of differential exon usage. The fact that antibodies to perlecan consistently identify a single precursor product of 400–450 kDa [9,15–17], strongly favours the existence of a single gene for this proteoglycan family. The multidomain nature of perlecan is reminiscent of other modular proteins which are often constituents of the extracellular matrix and may result from a mosaic blend of exons with diverse origins [18].

MOLECULAR ORGANIZATION: THE GENE, THE PROTEIN AND THE FIVE MODULES OF PERLECAN

Elucidation of the complete cDNA sequences of the mouse [1], human [19,20] and nematode [21] perlecan, together with the complete structural characterization of the human perlecan gene [22], have provided us with the detailed structural picture of the molecule which we possess today, and have clearly shown that the primary design of perlecan encompasses discrete molecular domains (Figure 1). There is a remarkable preservation of individual domains, each largely composed of a subset of exons flanked on either side by introns in the same phase, i.e. interrupting the coding sequence between codon triplets or after the first or second nucleotide. This homogeneity in codon phasing constitutes a powerful mechanism for the coupling of functional units via intronic recombination, thereby preserving a continuous open reading frame. The most striking feature of perlecan, in

Abbreviations used: LDL, low-density lipoprotein; LRP, LDL-receptor-related protein; N-CAM, neural cell adhesion molecule; EGF, epidermal growth factor; EHS, Engelbreth–Holm–Swarm; RFLP, restriction-fragment length polymorphisms; RT-PCR, reverse transcriptase-polymerase chain reaction; bFGF, basic fibroblast growth factor.

* To whom correspondence and reprint requests should be addressed.

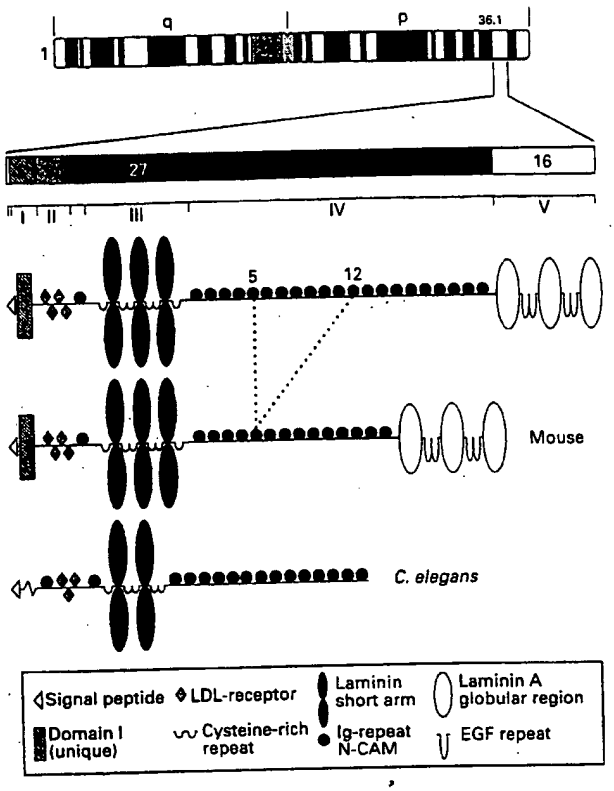


Figure 1 Schematic representation of human chromosome 1, the exonic organization of human perlecan gene and the deduced protein structure from human, mouse and nematode *C. elegans*

The distinct domains of the human perlecan gene are represented by the differently coloured rectangles containing the number of exons. The corresponding protein modules are designated by the Roman numerals underneath, and their colour-coded symbols are shown at the bottom of the figure.

addition to its colossal dimensions, is the presence of numerous internal repeats and the montage of modular protein domains. These modules harbour protein motifs with domain similarities to known polypeptides involved (i) in the binding and delivery of nutrients and lipids, (ii) in cellular growth and (iii) in intercellular signalling pathways.

The human perlecan gene (*HSPG2*) resides on the telomeric region of chromosome 1

Previous studies have provided evidence that a single gene is present in the haploid human [22–25] and mouse [26] genomes. The first evidence for the presence of perlecan on the telomeric region of human chromosome 1 was obtained by cross-species *in situ* hybridization [23] using as the probe a mouse cDNA encompassing the neural cell adhesion molecule (N-CAM) domain of perlecan [10]. This localization was subsequently confirmed by two independent studies using human probes under highly stringent conditions and employing human/rodent somatic cell hybrids containing partial deletions of chromosome 1 [24] and by *in situ* hybridization analysis [25]. Both studies clearly showed that the perlecan gene, whose genomic designation is *HSPG2*, is located within a narrow region of the telomeric portion of chromosome 1 (Figure 1), with the most likely primary site being the 1p36.1 band. It is noteworthy that the mouse perlecan gene has been localized to chromosome 4 adjacent to the alkaline phosphatase-2 gene [26]. Because the same gene is also present in human 1p36.1-p34 [27], there is strong evidence for the preservation of a syntenic region between human chromosome 1 and mouse chromosome 4. Although no disease has been assigned to an abnormality in the perlecan gene locus, there are numerous reports involving either deletions or loss of heterozygosity of the short arm of chromosome 1 in familial cutaneous melanoma [28,29], neuroblastoma [30,31], multiple endocrine neoplasia type 2 [32], breast carcinoma [33] and colorectal cancer [34]. In addition, it has been proposed that the distal portion of chromosome 1 may contain one, or several, tumour suppressor genes [33]. Since several restriction-fragment length polymorphisms (RFLP), generated with either *Taq*I/*Eco*RI [24] or *Bam*HI [25], have been identified in the human perlecan gene, these RFLPs could be useful in linkage of this locus with diseases connected to the perlecan gene.

The promoter of perlecan

Transcription of the perlecan gene is driven by a promoter which is highly enriched in GC content, with over 80% GC in the 500 bp immediately upstream of the leader exon (Figure 2). It has a CpG/GpC ratio of approx. 0.9, thus indicating the absence of CpG suppression [35]. The promoter contains four GC boxes and three GGGCGG hexanucleotide sequences which are known binding sites for the zinc-finger transcription factor SP1 [36]. Moreover, five GGGCGG hexanucleotide sequences and a GC-box were found in the first exon and first intron. This region also contained 21 CCGG tetranucleotide sequences, which is the recognition site for the methylation-sensitive *Hpa*II enzyme. The more distal part of the perlecan promoter contained two viral-enhancer AP2 motifs and three short palindromic direct and indirect repeats, which by forming a secondary structure could influence the regulation of perlecan gene expression. These features, together with the lack of canonical TATA or CAAT boxes, are often associated with multiple transcription start sites. Indeed, both primer extension and S1 nuclease mapping have demonstrated at least five distinct transcription start sites [22]. In addition to these characteristics, the 5′ end of the perlecan gene and its promoter are found in a CpG island, also referred to as a *Hpa*II tiny-fragment island [35]. Such DNA regions contain clusters of unmethylated CpG dinucleotides, a microenvironment in which the number of CpG approximately equals the number of GpC [35]. These islands have been correlated with transcriptional control regions and are typically observed in genes that encode oncoproteins, growth factors, transcription factors and housekeeping proteins. This is in agreement with the ubiquitous nature and the constitutive expression of perlecan in most tissues. Similar promoter features are observed in several extracellular matrix genes and cell-adhesion-molecule genes, including those encoding human laminin B1 [37] and B2 [38] chains, and N-CAM [39]. The promoter regions of these genes also exhibit a high GC content and contain SP1-binding sites as putative regulators of transcription (Figure 2). The similarities among these molecules, from promoter to gene arrangement and protein structure, suggest common regulatory mechanisms and therefore functional significance.

Domain I: a module unique to perlecan

Following the signal peptide, there is a small domain of 172 amino acids that contains three consecutive Ser-Gly-Asp (SGD)

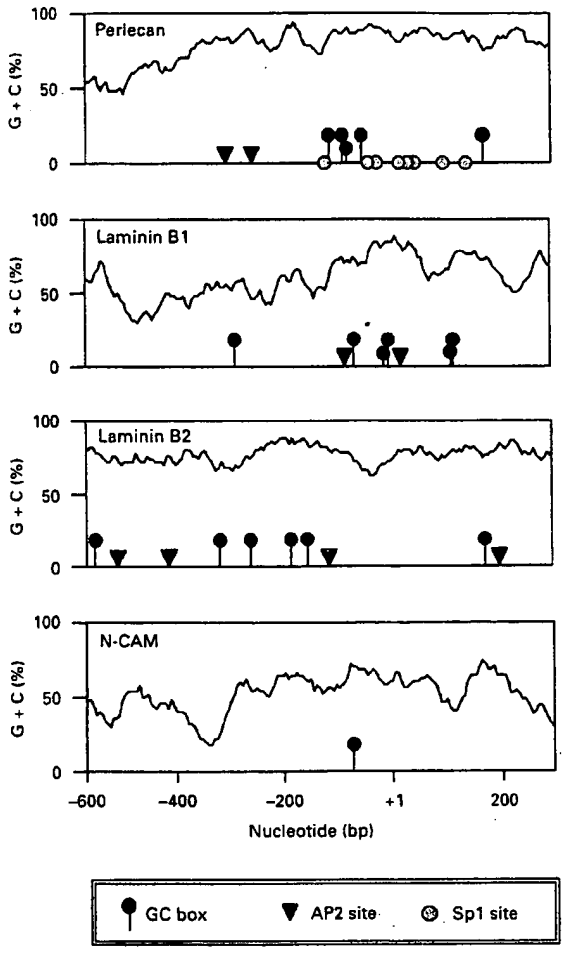


Figure 2 Comparison between the relative G+C composition in the promoter region of the human perlecan gene and that of the related proteins laminin B1, laminin B2 and N-CAM

Each panel shows the percentage of G+C content in the first 600 bp of promoter and approx. 250 bp of the 5′ end of the designated genes. The G+C content was determined from a window of 50 bp. The symbols of the various *cis*-acting elements are summarized in the diagram at the bottom of the figure.

Gene	Sequence	Reference
Human perlecan	DSI[redacted]GDLG[redacted]LG[redacted]FQM	[19, 20]
Mouse perlecan	EDA[redacted]GGLG[redacted]VG[redacted]FQM	[1]
Syndecan-1	EDQD G[redacted]DS PAAE G[redacted]QD FTFE T[redacted]NT	[43]
Syndecan-3	PVSGGP[redacted]FE	[44]
Syndecan-4	EFEL[redacted]LE	[142]
Betaglycan	GYEDLA[redacted]DNG	[143]
Glypican	PQAE I[redacted]HL EG[redacted]GC	[45]

Figure 3 Potential heparan sulphate attachment sites in perlecan and other heparan sulphate proteoglycans

Comparison of the putative heparan sulphate attachment sites in domain I of human and mouse perlecan and similar potential GAG attachment sites in human syndecan-1, rat syndecan-3 (N-syndecan), human syndecan-4 (amphiglycan), rat betaglycan (transforming growth factor-β type-III receptor) and human glypican. Most of the sequences comply at least in part with the proposed GAG attachment consensus sequence (D/E)GSG(D/E). Ser-Gly sequences are underlined and shown in dark red, while flanking acidic residues are shown in light red. Some gaps have been introduced into the sequences in order to optimize alignments.

tripeptides, the predicted site for attachment of the heparan sulphate side chains [1]. Whether this is the only domain capable of being substituted with glycosaminoglycans or just one of the possible glycosylation sites needs to be established by future experiments. It should be pointed out, however, that the perlecan protein core harbours over 50 Ser-Gly (SG) dipeptides, some of which are flanked by acidic amino acids and would comply with a proposed consensus sequence for a glycosaminoglycan attachment site [40,41]. The SGD-sequence region shares features with proposed glycanation sites in other heparan sulphate proteoglycans (Figure 3) and is most similar to sequences found in some of the syndecan family of cell-surface proteoglycans [42–45]. In contrast with the other domains, domain I does not harbour any internal repeats, lacks cysteine residues and is quite enriched in acidic amino acids. These physicochemical attributes render this domain significantly different from the others. Indeed, neither its amino acid nor its nucleotide sequence is related to any previously characterized entry in the available databases: it seems that this is the only domain unique to perlecan. Of note, is the exonic organization of this domain, inasmuch as the first SGD triplet is split between exons 2 and 3 [22]. Because these exons are separated by phase I introns at either end, it is plausible that exon 3 may be spliced out; a consequence of this would be the synthesis of a polypeptide without heparan sulphate side chains. That this event may occur is supported by the observation that colon carcinoma cells, under routine culture conditions, can release perlecan protein core into the medium without heparan sulphate side chains [9].

Collectively, these observations suggest that perlecan can also be a non-glycanated protein, a notion that has been corroborated by the recent detection of perlecan protein core in cartilage [46], and confirmed by our immunohistochemical data using a monoclonal antibody specific for domain III (cf. Figure 8). One fundamental question that needs to be addressed in future studies is to what extent this gene product is secreted as a glycoprotein rather than as a proteoglycan and what functional roles it may have during ontogeny and neoplastic growth.

Domain II: identity with the low-density lipoprotein (LDL) receptor

The second discrete domain of perlecan is homologous to the ligand-binding portion of the LDL receptor [47]. This region is encoded by three distinct exons, as is the LDL receptor gene, and contains four cysteine-rich motifs that are also shared by a number of binding molecules (Figure 4). The overall genomic organization of this domain is remarkably similar to that of the LDL receptor. For example, the first two cysteine-rich repeats of perlecan and the corresponding repeats in the LDL receptor are encoded by one exon of nearly identical size. The distal two repeats are also encoded by one highly conserved exon. More startlingly, all three exons are interrupted by phase I introns, thus allowing for the possibility of alternative splicing. It has been postulated that via alternative exon usage, the LDL receptor gene can generate multiple forms of the receptor with varying degrees of affinity for LDL [47]. The consensus sequence of the LDL- receptor repeat has six conserved cysteine residues spaced over an approx. 40-amino-acid region (Figure 4). It is iterated

Gene	Sequence	Copy number	Reference
Human perlecan	GPQEAA R-----NGH IPRDYL DGQED E[redacted]LD--	4	[19, 20]
Mouse perlecan	GPQEAS H-----SGH IPRDYL DGQED R[redacted]LG--	4	[1]
Nematode *unc-52*/perlecan	MADEKA G-----NNE VKNDYV DGEPD RDRSDEAN--	3	[21]
Complement C9	GND-FQ S-----TGR IKMRLR NGDND GDFSDEDD--	1	[48]
Heyman nephritis antigen	QPTQFR P-----DHR ISPLYV DGDKD A[redacted]G---	13	[49]
α_2-Macroglobulin receptor	GVDEFR K----DSGR IPARWK DGEDD G[redacted]PKEE	31	[50]
Nematode LRP	TPDQFK V----SSGL IPASWK DGQQD D[redacted]PKFG	35	[51]
Quail receptor for RS virus	PPGQFR SEPPGAHGE YPQDWL DGHPD DDGRDEWG--	1	[52]
Chick receptor for RS virus	SPGQFH SEPRDPQTD YPLEWL DGHPD DDGRDEWG--	1	[52]
LDL receptor consensus	F G I DG D [redacted]	7	[47]

Figure 4 Amino acid sequence similarity between perlecan domain II and the ligand motif of the LDL receptor and related proteins

The strong similarity in the second repeat of domain II of the human [19,20], mouse [1] and nematode [21] perlecan are compared with regions of similarity in complement C9 [48], the fourth repeat of the antigen glycoprotein, also known as GP330, which causes the Heymann nephritis [49], the fifth repeat of the α_2-macroglobulin receptor [50] also known as LRP, the twelfth repeat of the nematode LRP [51] and the avian receptors for the Rous sarcoma (RS) virus [52]. The consensus sequence of the human LDL receptor [47] is presented at the bottom. Alignment was obtained using the FASTP and ALIGN programs in the GCG and PCGENE packages respectively. The pentapeptide DGSDE, a proposed binding site for LDL, is underlined and coloured in dark red. The six conserved cysteine residues are shown in light red. Gaps, introduced to optimize alignment, are shown as dashes.

seven times and contains an additional 11 amino acids that define this LDL-receptor repeat [47]. Interestingly, mammalian perlecan contains the pentapeptide DGSDE, a sequence that mediates ligand binding by the LDL receptor [47]. One to several copies of this repeat are found in a number of molecules including mouse [1] and nematode [21] perlecan, complement C9 [48], the autoimmune target glycoprotein (also known as GP330) involved in Heymann nephritis [49], the α_2-macroglobulin receptor [50], the LDL-receptor-related protein (LRP) protein in *C. elegans*, a protein which appears to be the orthologue of the mammalian α_2-macroglobulin receptor [51], and the receptors for subgroup A of the Rous sarcoma virus in the quail and chicken [52]. Expression of these viral genes renders mammalian cells susceptible to Rous sarcoma virus infection [52], thus opening the possibility that viruses may exploit similar protein motifs to gain entry into host cells.

Perhaps one of the most interesting, yet speculative, functions of perlecan is the possible role of this pericellular proteoglycan in the metabolism of LDL. Because perlecan is expressed at high levels along the sinusoidal spaces of the liver (cf. Figure 7), spleen and in the subendothelial region of all blood vessels, it seems logical to speculate that LDL would be in contact with perlecan during its normal circulatory life as well as during endothelial denudation or in hyperlipidaemic states. Binding of lipids to the basement membranes and extracellular matrices of blood vessels is an established observation, and this property has been generally attributed to the glycosaminoglycan chains [53]; but no direct link between LDL binding and protein cores has been established. A plausible scenario would include the formation of a supramolecular complex between perlecan, either associated with the basement membrane or the cell surface, and LDL. This complex could conceivably be stabilized by the interaction of the closely clustered heparan sulphate chains that are known to bind apolipoproteins [54]. Interestingly, the extracellular concentration of LDL can in turn modulate the expression of perlecan in cultured endothelial cells [55]. An alternate view would be to consider domain II simply as providing a sorting mechanism for the polarized secretion of perlecan to the basolateral region as proposed for mammary epithelial cells [14]; however, perlecan can be localized around tumour cells without any apparent vectorial secretion but still in close association with the plasma membrane [56].

Domain III: homology with the short arm of laminin A chain

Domain III exhibits extensive similarity with the internal segments of laminin [57,58], particularly with the region of the A chain that comprises one of the short arms of the laminin multimer [58]. This region consists of three distinct globular subdomains, characteristically devoid of cysteine residues, which are flanked on either side by cysteine-rich extended domains. The globular regions have an overall amino acid configuration which resembles that of two subregions of the laminin A chain, the IVa and IVb subdomains [58], and to a lesser extent the laminin B2 subdomain IV (Figure 5). The rod-like segments are composed of a series of repeats, each typically harbouring eight highly conserved cysteine and two glycine residues arranged in essentially the same pattern as that of laminin domains III or V. The entire domain is encoded by 27 exons ranging in size from 50 to 231 bp. Interestingly, there is no apparent correlation between the exon arrangement and protein subdomains, a phenomenon that occurs in all the related genes [22]. This considerable divergence suggests that the laminin portion of the perlecan gene might have evolved from an ancestral gene enduring significant exon shuffling. We have also found [22] a duplication of the distal portion of exon 30 in a genomic region adjacent to exon 23. This duplicated exon, although not yet formally discovered in any mammalian mRNA, could conceivably be spliced out, thereby forming a truncated perlecan species. Another feature of this human perlecan domain is that it lacks the RGD triplet, a sequence that is present in the mouse species and may be involved in cell-surface binding (see below).

Domain IV: homology with the immunoglobulin superfamily and N-CAM

Domain IV has two distinguishing features: (i) it is the largest domain of human perlecan, encoding a polypeptide of over 200 kDa; and (ii) it is the most iterative domain, containing 21 consecutive repeats typically observed in the members of the

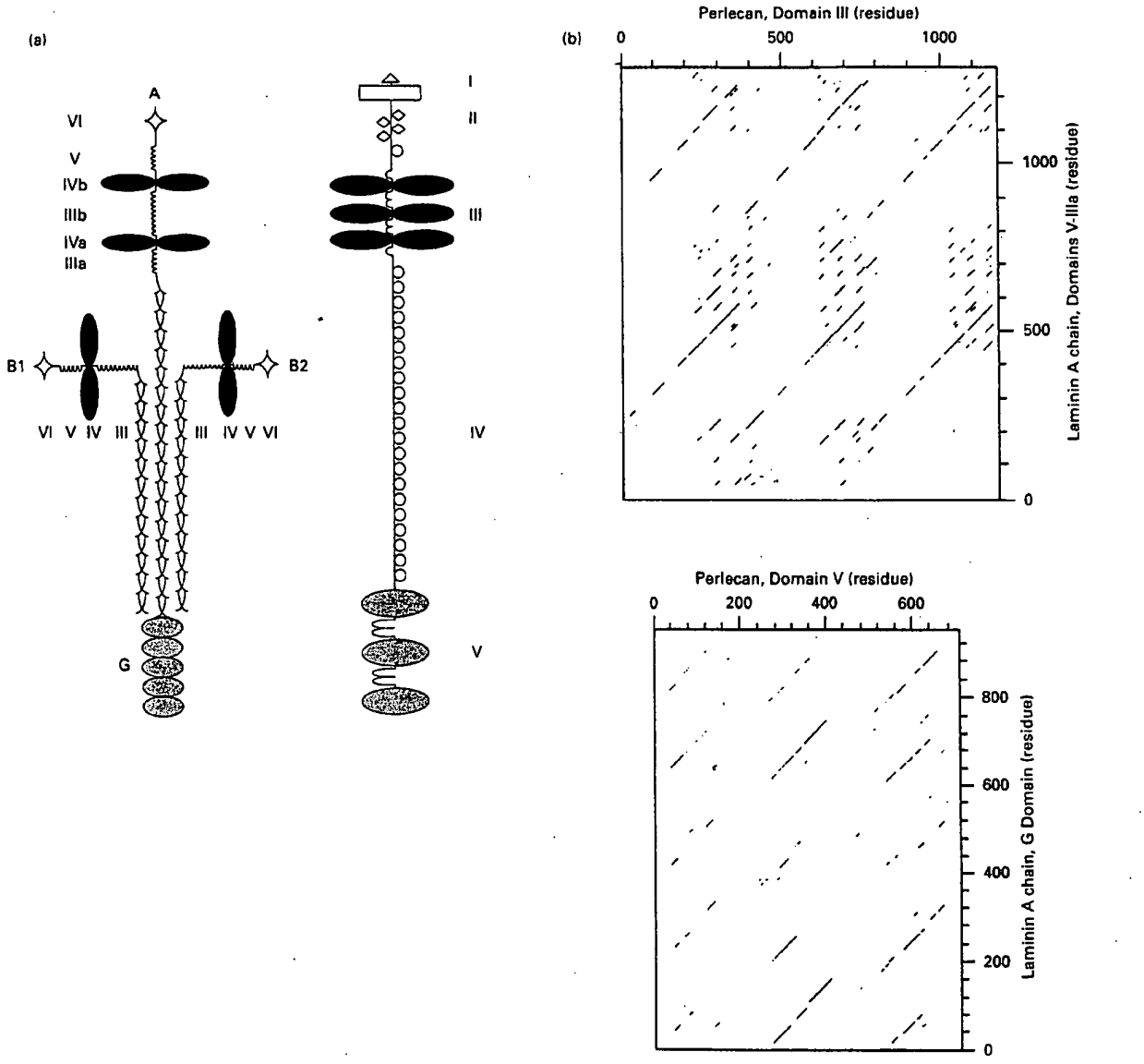


Figure 5 (a) Schematic representation of human laminin and perlecan and (b) homology plot analysis of the respective homologous domains

(a) The three chains of laminin are represented with their distinct subdomains. Subdomain IV and the G-domain of laminin are colour-coded in dark red and light red respectively, to indicate the homologies with the perlecan protein core shown on the right. Of note, a recent report [141] has proposed a new nomenclature for the heterotrimeric forms of laminin. Accordingly, the laminin isoform depicted in (a) would be called laminin-1 and the three chains A, B1 and B2 would be designated as $\alpha 1$, $\beta 1$ and $\gamma 1$ respectively. (b) The 1286 amino acids of subdomains V to IIIa of the A chain of laminin were compared with the 1172 residues of perlecan domain III (upper panel), and the 950 amino acids of the G-domain of laminin were compared with the 1005 residues of perlecan domain V (lower panel). Homology-plot analyses were done using the Dot-Plot program (GCG package) with a window setting of 30 and a stringency of 15. A single dot indicates that > 15 residues match in a searching area of 30 amino acids.

immunoglobulin gene superfamily [59]. In contrast with the human, the mouse and nematode perlecan species contain only 14 repeats (Figure 1). This domain is encoded by 40 exons and harbours the largest collection of Ig repeats so far described. Similar domains are shared by all members of the Ig gene superfamily, which includes molecules involved in immune recognition as well as molecules with no immunological function. This structural unit is characterized by a primary amino acid sequence of 70–100 residues with an essentially invariant disulphide bridge spanning 50–70 residues, and numerous conserved glycine and tryptophan residues [19]. Specifically, the 21 consecutive Ig repeats resemble the organization of the N-CAM [59], which is an established member of the Ig superfamily. With the exception of repeats 17–21, each Ig repeat is encoded by two exons, thus supporting the proposal that the Ig superfamily was founded on an ancestral block of two exons [59]. The sequences that exhibit the strongest internal sequence similarity follow a strict pattern; specifically, the first half of the repeat is always interrupted by a phase 0 intron after the dipeptide His-Glu, whereas the other half of the repeat is invariably interrupted by

a phase I intron. This arrangement makes this region an optimal candidate for alternative exon usage or skipping (see below). It is noteworthy that in the mouse [1], the first and the twelfth Ig repeats contain an extra cysteine which may be involved in disulphide bonding and would thus generate a more compact, globular N-CAM region [1,60]. The same possibility could occur for the human species inasmuch as these two cysteine residues, although located much further apart because of the intervening Ig-like repeats (Figure 1), are still fully preserved [19]. These data are consistent with the multiple globular domains observed in rotary-shadowed pictures of isolated perlecan molecules from the EHS tumour matrix [8,61]. The main function of this module is still conjectural. By analogy with other molecules containing the same motifs [62], this module could: (i) stabilize homophilic protein–protein interactions; (ii) promote dimerization of perlecan molecules within the basement membrane, thus contributing to the stabilization of this matrix; or (iii) enhance the adhesion of neighbouring cells carrying perlecan at their surfaces.

Domain V: homology with the C-terminus of laminin A chain

This protein module comprises the C-terminal end of the mammalian species of perlecan, but surprisingly is missing in the nematode orthologue (Figure 1). Domain V is encoded by 16 exons and composed of three globular and four epidermal growth factor (EGF)-like motifs, exhibiting structural similarity with the C-terminal, globular region of the laminin A chain [58] (Figure 5). As in the case of domain III, the other region with sequence similarity to the laminin molecule, domain V, also lacks correlation between subdomain boundaries and exon structure, indicating that the two laminin-like regions of perlecan have diverged from the ancestral gene quite significantly during evolution. As previously proposed [63], the relationship of exons to protein structure probably represents a degenerate state of ancestral correspondence between exons and structure/function modules of proteins. The four EGF-like repeats of perlecan domain V are composed of about 40 amino acids each, with a consensus sequence typically found in EGF type-I repeats [64] and several conserved glycine residues that may be involved in the proper folding of this region. It is noteworthy that the presence of alternating globular and EGF-like domains has now been recognized in a number of molecules that are involved in cell adhesion and growth. For example, significant structural similarity exists between the C-termini of perlecan and neurexins; the latter comprise a large family of polymorphic cell-surface proteins, as well as secreted variants [65,66]. The repeats of the three distinct species of neurexin (Iα, IIα, and IIIα) show a similar degree of homology to agrin, a component of the basement membrane that causes the aggregation of acetylcholine receptors [67], as well as to laminin [57,58] and perlecan [1,19,20]. Interestingly, in the case of neurexin IIIα [66], the carboxyl end can undergo extensive alternative splicing events with the potential of generating a membrane-bound and a truncated form, suggesting that some forms of neurexin IIIα may be secreted. The terminal domain of perlecan could be involved in both homotypic and heterotypic interactions, as in the case of the mouse EHS proteoglycan [61,68]. It could also be involved in the modulation of cellular growth via the EGF repeats, inasmuch as comparable EGF-like repeats of mouse laminin can induce cellular growth in cultured cells possessing EGF receptors but not in cells lacking the receptor [69]. In line with this view is the fact that synthetic peptides from the globular G domain of laminin can promote cell adhesion and neurite outgrowth, and can interact with heparin and the β1 integrin subunit [70]. Domain V contains two SGxG tetrapeptides which may be substituted with glycosaminoglycan side chains other than heparan sulphate. It is noteworthy that hybrid forms of perlecan containing both chondroitin and heparan sulphate side chains have been described recently in a cell line derived from the EHS tumour [71] and in human corneal fibroblasts [72]. In addition, a hybrid dermatan/heparan sulphate proteoglycan from human placenta [73] has been shown to be immunologically related to human perlecan [19].

Finally, domain V contains two conserved Leu-Arg-Glu (LRE) tripeptides that have been invoked as the primary sites for the binding of motor neurons to s-laminin in the synaptic basement membrane [74]. Neurons bind to immobilized LRE-containing peptides, and soluble LRE sequences block attachment of neurons to s-laminin fragments [74]. The presence of two LRE motifs in human perlecan, in contrast with the mouse form where only one is conserved [1], suggests that these motifs may play important roles in perlecan-dependent neurite outgrowth.

WIDESPREAD EXPRESSION OF PERLECAN: FUNCTIONAL IMPLICATIONS

Perlecan is one of the earliest gene products encoding basement membrane constituents to be expressed in embryonic life: it can be detected at the 2–4-cell stage of the mouse embryo, and is later deposited in all embryonic basement membranes [75]. Interestingly, perlecan is found at the exterior surface of the trophectoderm during the attachment phase of blastocyst implantation *in vivo*, an expression that correlates with the acquisition of attachment competence by mouse embryos *in vitro* [76].

The cell- and tissue-specific expression of perlecan is now being re-evaluated inasmuch as a number of epithelial and mesenchymal cells are found to express the perlecan message. For example, both colon carcinoma cells and fibroblasts express a single approx. 14 kb transcript (Figure 6, lanes 1 and 2 respectively), and several perlecan domains, including I, II and IV, can be detected by reverse-transcriptase polymerase chain reaction (RT-PCR) in colon carcinoma, osteosarcoma, fetal lung and skin fibroblastic, and prostate carcinoma cells (Figure 6, lanes 3–10). Similarly, *in situ* hybridization analyses have demonstrated perlecan message in a variety of cells independent of whether they assemble a classical basement membrane [19,77]. A number of studies have localized perlecan to various basement membranes and pericellular regions [5,19,71,75,77–83]. We have recently developed a monoclonal antibody that recognizes a recombinant fusion protein containing domain III of human perlecan and have investigated systematically its expression in human tissues [77]. Perlecan is present not only in the basement membrane zone of every vascularized organ, but also in the tumour stroma of several human cancers (Figure 7). Of interest is an abundant deposition of perlecan in the perisinusoidal space of the liver (Figure 7b), as well as in the sinusoidal spaces of all the lymphoreticular and endocrine organs investigated [77]. Because of the large blood flow through these tissues, perlecan would be continuously exposed to circulating blood elements and immunocompetent cells.

It is now becoming apparent that perlecan is diffusely present in the pericellular matrices of a number of organs, and that the fibroblast, a cell type that does not assemble a traditional basement membrane, is a main producer of the proteoglycan. For example, *in situ* hybridization studies of human skin [77] have demonstrated perlecan message in the fibroblasts of the upper dermis but not in keratinocytes. Perlecan has been localized to the dermo-epidermal basement membrane of fetal skin during the first trimester of life, and as early as 54 days of intra-uterine development [80]. The presence of perlecan at the earliest time at

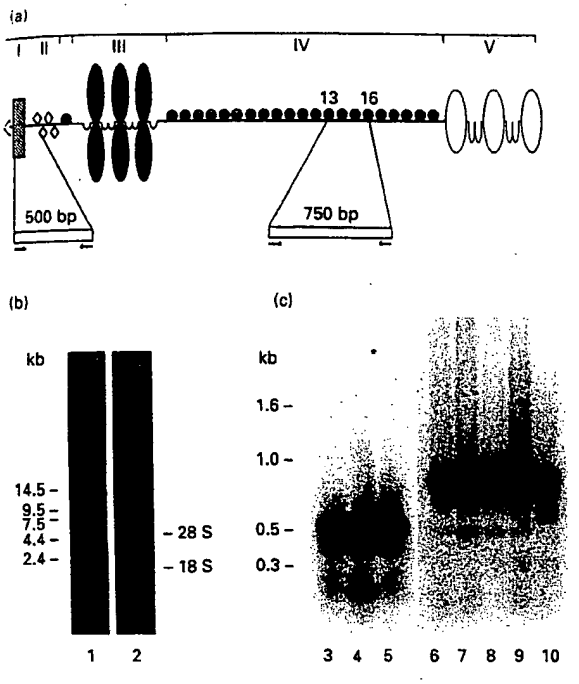


Figure 6 Expression of perlecan in normal and neoplastic human cells

A schematic representation of the perlecan protein structure and the various domains (roman numerals) is shown in (a). The arrows indicate the sense and antisense primers used in RT-PCR to amplify a 500 bp fragment in domain I–II, and a 750 bp fragment in domain IV, a region contained between Ig repeats 13 and 16 respectively. (b) and (c) show Northern blotting (lanes 1 and 2) and Southern blotting of RT-PCR products amplified from domain I–II (lanes 3–5) or domain IV (lanes 6–10). The following cellular RNAs were analysed: WiDr colon carcinoma cells (lanes 1 and 6), neonatal skin fibroblasts (lane 2), MG-63 osteosarcoma cells (lanes 3 and 8), CRL-1262 embryonic lung fibroblasts (lanes 4 and 9), PC-3 prostate carcinoma cells (lanes 5 and 10), and IMR-90 embryonic lung fibroblasts (lane 7). For Northern blotting, approx. 20 μg of total RNA was separated in a denaturing formaldehyde gel, transferred to a nylon membrane and hybridized under high stringency with a PCR-generated perlecan probe encompassing part of domain IV. For Southern blotting, the cDNAs amplified by RT-PCR were separated by analytical agarose gel electrophoresis, transferred to a nylon membrane and hybridized with PCR-amplified probes corresponding to the same regions of approx. 500 and 750 bp respectively. The Northern blotting was exposed for 48 h, while the Southern blotting was exposed for approx. 30 min.

which basement membrane is formed, supports the hypothesis that this large proteoglycan contributes to the early structural integrity of the epidermal–connective tissue interface [80]. In the developing intestine, by contrast, perlecan is manufactured by epithelial cells and is subsequently assembled into a basement membrane which includes the mesenchyme-derived collagen type IV [84]. Together, these findings suggest that basement membranes are the product of both ectoderm and mesoderm and that co-operation between different cells is necessary for correct orientation and assembly into a functionally complete basement membrane.

There has long been a controversy regarding the intrabasement membrane topology of perlecan. Earlier immunoelectron microscopic studies [85] have shown that the major heparan sulphate proteoglycan of renal glomeruli is restricted to the lamina rara interna; this corresponds to the concentration of polyanionic sites [86]. It is now felt, however, that this antigen probably represents a proteolytically processed form of perlecan, inasmuch as other studies have shown a diffuse presence of perlecan throughout the basement membrane [82,87]. Further support for this view is offered by the observation that monoclonal antibodies directed toward an epitope of perlecan [88] stain only the lamina densa on the stromal side of the basement membrane, thus indicating a vectorial insertion of perlecan. This view has the N-terminus with the heparan sulphate chains near the plasma membrane, whereas globular domain V would reach across the basement membrane and could interact with other extracellular matrix molecules. This orientation is reminiscent of that of laminin, where the short arms are considered to be buried in the basement membrane, whereas the long arm can traverse the full thickness [68]. Double tracks of approx. 4.5 nm width, visualized in the glomerular basement membrane and Reichert's membrane, have been interpreted as representing submicroscopical images of perlecan [89,90]. In human colon carcinoma cells, perlecan is localized to the cell surface [11] and can be acylated with both myristate and palmitate, two long-chain fatty acids [91]. This post-translational modification may contribute to the binding and vectorial secretion of perlecan under the appropriate circumstances.

In the liver, perlecan is expressed primarily by the sinusoidal endothelial cells but not by the hepatocytes. However, when hepatocytes are cultured *in vitro* they gain the ability to express perlecan [81], implying a central role of perlecan for cellular survival and growth. A traditional dogma needs also to be revised insofar as immunoreactive perlecan is found in normal articular cartilage (Figure 8a) and abundant deposits are seen in human chondrosarcomas (Figures 8c and 8d). This observation is provocative because cartilage is an archetypal tissue that does not contain blood vessels and does not assemble a basement membrane. The question is why a gene product as complex and heterogeneous as perlecan is expressed in these tissues. The answer probably lies in the fact that a significant proportion of perlecan can escape post-translational modifications and can be secreted as a glycoprotein, i.e. lacking the heparan sulphate side chains [9]. This view has now been confirmed experimentally in rat chondrosarcoma cells by the finding of a perlecan protein core that, instead of being converted into a fully glycanated proteoglycan, is released without attachment of the glycosaminoglycan side chains [46]. This product appears to be localized in the pericellular environment of the chondrocyte and to be involved in the attachment of these cells to their own substratum [46].

In summary, several generalizations can be drawn from the studies of perlecan expression discussed above: (i) perlecan appears to be more ubiquitous than previously considered, with a distribution that is not rigorously limited to epithelial/mesenchymal interfaces; (ii) epithelial/mesenchymal cell co-operation is required for full perlecan expression; (iii) qualitative and quantitative expression of the mature proteoglycan is tissue-specific; and (iv) post-translational modifications of perlecan, and especially the attachment of heparan sulphate side chains, can be bypassed under certain tissue-specific conditions.

BINDING PROPERTIES OF PERLECAN

Binding to extracellular matrix proteins: homotypic and heterotypic interactions

The discovery that this large, multimeric macromolecule can self-assemble into a dynamic structure implies that perlecan is directly involved in the formation of basement membranes in living organisms [61,68]. This realization surfaced from key experiments [61] which showed that perlecan can self-aggregate into dimeric or multimeric forms when incubated under neutral isotonic

conditions. This homotypic interaction is apparently mediated by the carboxyl end of the perlecan protein core, or at least the end opposite to the glycosaminoglycan-binding region, the heparan sulphate chains appearing peripherally oriented [61]. Intriguingly, a recent report suggests that in EHS tumour perlecan, the heparan sulphate chains may be located at the carboxyl end [92]. The evidence for this is indirect and inferred from peptide sequencing and size estimation of proteolytic fragments obtained from purified EHS perlecan; however, no peptide sequence could be obtained from the fragment shown to be carrying the heparan sulphate chains [92]. Direct biochemical evidence is, therefore, needed to establish the precise site of glycosaminoglycan binding and clarify these seemingly contradictory results. In addition to these homotypic interactions, a number of proteoglycans, which have now been recognized as perlecan, have been involved in heterotypic interactions with numerous extracellular macromolecules including laminin [68,93], nidogen [93,94] and fibronectin [73,95]. Earlier workers have observed that, in order to purify and extract perlecan from the EHS tumour, denaturing solvents and chaotropic agents such as guanidinium chloride were required [5,6,96]. This indicates that perlecan is tightly bound to other matrix macromolecules and that both the protein core and the polyanionic chains might be involved in these interactions. For example, during EHS tumour purification, a significant proportion of laminin remains associated with perlecan and is removed only after CsCl gradient centrifugation [68]. Another basement membrane molecule that can interact with perlecan is nidogen [93]. Nidogen binds the protein core of perlecan via amino acid sequences mapped to the G2 domain [94], a region that also harbours binding sites for collagen type IV. It has been speculated that nidogen, via its laminin-binding region in the globular G3 domain together with the G2-binding region, may modulate the formation of a supramolecular complex among perlecan, laminin and collagen type IV in a heparan sulphate chain-independent fashion [97].

A large heparan sulphate proteoglycan from human fibroblasts binds fibronectin, thereby functioning as mediator of fibronectin–cell surface interaction in fibroblasts [98] and perhaps other cell types [73], and this interaction may anchor the ubiquitous fibronectin to certain basement membranes. This proteoglycan has been subsequently shown to be identical with human perlecan by comparison of its peptide sequence with the amino acid sequence deduced from cDNA cloning [19]. Intriguingly, a basement membrane heparan/dermatan sulphate hybrid proteoglycan, which was isolated from human placenta [73], also appears to be identical with perlecan [19]. This proteoglycan also binds fibronectin, apparently via covalent interactions, and may contribute to some of the fundamental functions of fibronectin in cell adhesion and spreading [73].

Binding to cell surfaces

About a decade ago, we reported that human colon carcinoma cells synthesize a surface proteoglycan which cross-reacted with anti-perlecan antibodies [11]. Later, it was unequivocally shown by cDNA cloning to be perlecan [24]. This colon carcinoma perlecan is closely associated with the plasma membrane: it is localized to the microvillar surface [56], the side opposite to the basement membrane; it cannot be displaced by high concentrations of heparin; and it requires detergent or mild trypsinization for its efficient removal from the cell surface [11]. We interpreted these data as being indicative of an intercalated proteoglycan sharing immunological and structural features with the basement membrane proteoglycan purified from the mouse EHS tumour [5]. These observations can now be partly reconciled with the notion that perlecan may be closely linked to the cell surface via receptor proteins recognizing the protein core and possibly stabilized by the heparan sulphate chains. Earlier studies, in fact, have proposed that perlecan can bind cell-surface receptors, originally shown to be of a size smaller than usual integrins [99]. More recent investigations, however, have clearly shown that the attachment and spreading of several tumour cells on a perlecan substratum is mediated by $\beta 1$ integrins, but not by the $\alpha 6\beta 1$ receptor typically observed for laminin [92]. Aortic endothelial cells, which synthesize and deposit perlecan [13], interact with the protein core of perlecan via $\beta 1$ and $\beta 3$ integrins, and this binding is partially RGD-dependent [100]. Apparently, this interaction is sensitive to a cell-mediated effect of heparin and heparan sulphate which, therefore, may act as regulatory ligands [100]. The cell-adhesive properties of perlecan were assigned to an approx. 160-kDa fragment which was localized to the C-terminus of the proteoglycan, a region containing part of the N-CAM repeats and domain V [100]. Note, however, that human perlecan does not contain an RGD sequence and therefore these findings may not be relevant to the human species.

Binding to growth factors

Recent discoveries in the field of growth factor and cytokine research have provided novel information regarding the role of pericellular heparan sulphate proteoglycans in the storage, binding and delivery of such compounds [95,101,102]. A cluster of important papers have reported a close association between basic fibroblast growth factor (bFGF), which is a heparin-binding angiogenic protein [95], and basement membrane constituents including mouse perlecan [103–107]. The bFGF appears to bind heparan sulphate chains and is released from the bovine cornea subendothelial matrix by heparin or heparinase, but not by chondroitin sulphate or chondroitinase [103]. It has been proposed that the sequestration of angiogenic endothelial cell mitogens in the basement membrane may be a general mechanism for regulating accessibility of these substances to the vascular endothelium [103]. Abnormal release of bFGF during wound healing or cancer invasion may contribute to local tissue neovascularization [108]. Interestingly, both heparinases I and III inhibit neovascularization *in vivo* and proliferation of capillary endothelial cells mediated by bFGF *in vitro* [109]. Because approx. 70% of bFGF added to capillary endothelial cells binds to the extracellular matrix, while only 7% binds to the cells [110], it is likely that specific sequences in the heparan sulphate chains covalently attached to perlecan protein core are directly implicated in the control of angiogenesis. In agreement with this view, is the discovery of a high affinity ($K_d \approx 30$ nM) between iodinated bFGF and EHS tumour perlecan [104]. This binding, at least in endothelial cells, seems to protect bFGF from proteolytic degradation [106], and bFGF–heparan sulphate complexes can be released from these cells via a plasminogen activator-mediated proteolysis [107]. High-affinity ($K_d \approx 1.5$ nM) binding of interferon-γ to heparan sulphate chains of an EHS tumour-derived extract has also been reported [111]. Finally, a recent study has shown an interaction between transforming growth factor-β and perlecan in the basement membrane of mouse endodermal cells [112], an intriguing observation inasmuch as this growth factor can in turn modulate the expression of perlecan in colon carcinoma cells [113].

The mechanism of action of bFGF at the cell surface has been partly elucidated by studies involving mutant cell strains lacking the enzymes necessary for the biosynthesis of heparan sulphate chains. Upon transfection of the FGF receptor, the defective

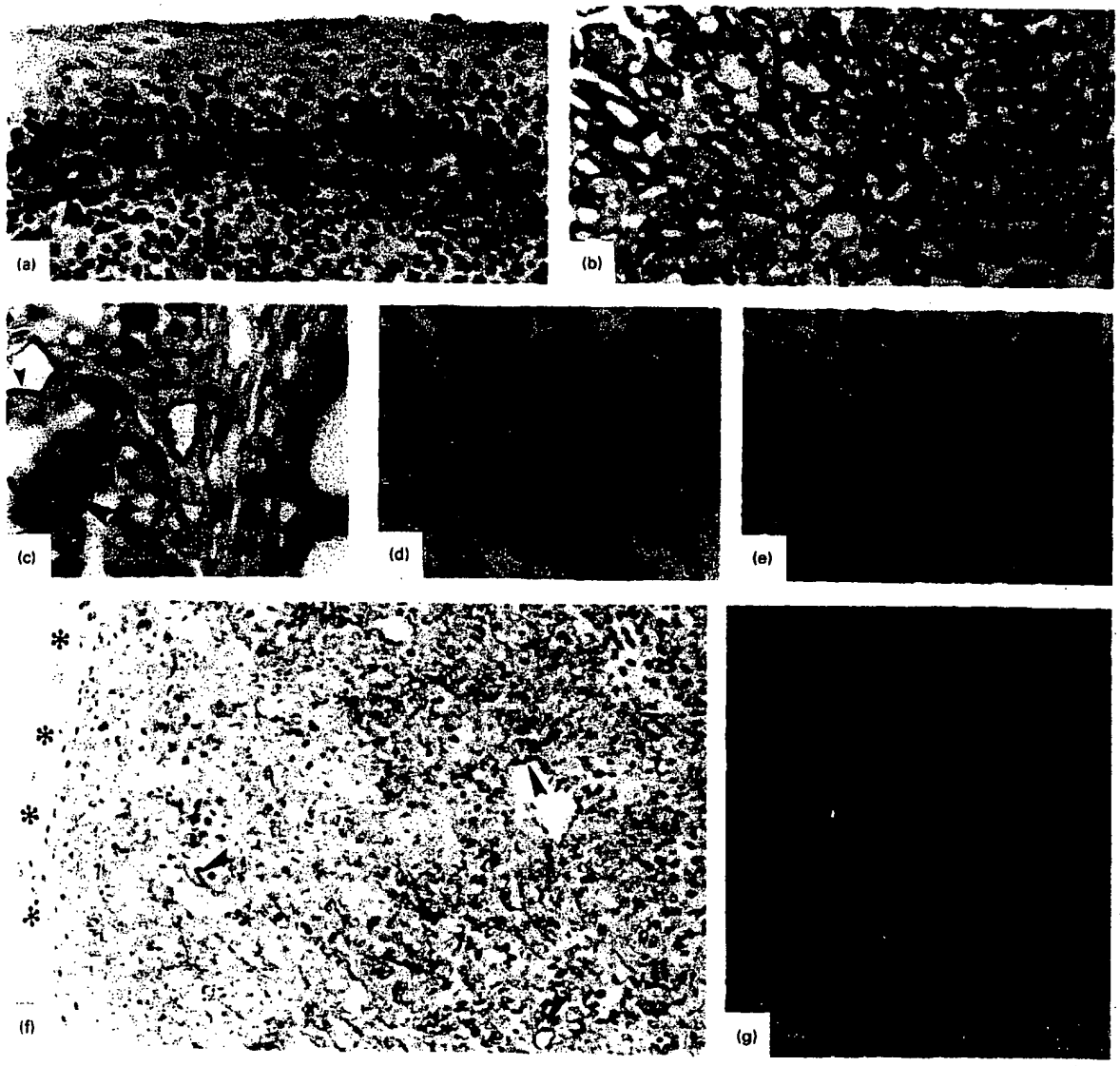


Figure 7 Immunolocalization of perlecan in human tissues using a monoclonal antibody against a recombinant fusion protein containing domain III of human perlecan protein core

The monoclonal antibody 7B5 was shown previously to react by Western blotting exclusively and specifically with the laminin-like domain III, a fusion protein generated in a prokaryotic expression system, and not to cross-react with the mouse perlecan [77]. The human perlecan epitope (red staining) is present in the basement membrane of oral mucosa (**a**, arrowheads), in the perisinusoidal space of the liver (**b**), in the basement membranes of endothelial cells (**c**, small arrowhead) and prostate epithelium (**c**, larger arrowhead), but can also be observed as diffuse deposits within the tumour stroma of breast (**d**) and colon (**e**) cancers. (**f**) shows a tumour xenograft 2 weeks after subcutaneous injection of approx. 1×10^6 PC-3 prostate carcinoma cells in a nude SCID mouse. Notice the positive reaction around small blood vessels (arrowheads), which are of mouse origin, and around the human tumour cells. In contrast, the basement membrane zone of the mouse skin (asterisks) is totally unreactive in agreement with the lack of cross-reactivity of our monoclonal antibody. (**g**) is a higher magnification showing intense reactivity around newly formed tumour microvessels (arrowheads). [Original magnification: (**a**) and (**b**), × 350; (**c**–**e**), × 450; (**f**), × 250; (**g**), × 600.]

cells could not bind bFGF unless supplemented with exogenous heparin or heparan sulphate [114]. In contrast, the wild-type cells were capable of binding bFGF without the need of exogenous glycosaminoglycan.

Subsequently, heparin-dependence was shown both for the interaction of bFGF with a recombinant FGF receptor species and for bFGF-induced mitogenesis [115]. An intuitive explanation for this phenomenon is that binding of the growth factor to the glycosaminoglycan may alter the conformation of bFGF, thereby facilitating its interactions with the receptor. The specific binding site for bFGF has now been identified as a unique carbohydrate sequence within the heparan sulphate chains purified from the [^{3}H]glucosamine-labelled total proteoglycan pool synthesized by skin fibroblasts [116]. Using bFGF-affinity chromatography, a heptad-saccharide containing both N-sulphate and iduronic acid 2-sulphate residues was shown to be essential for binding activity to bFGF [116]. Although it is not known whether extracellular matrix heparan sulphate proteo-

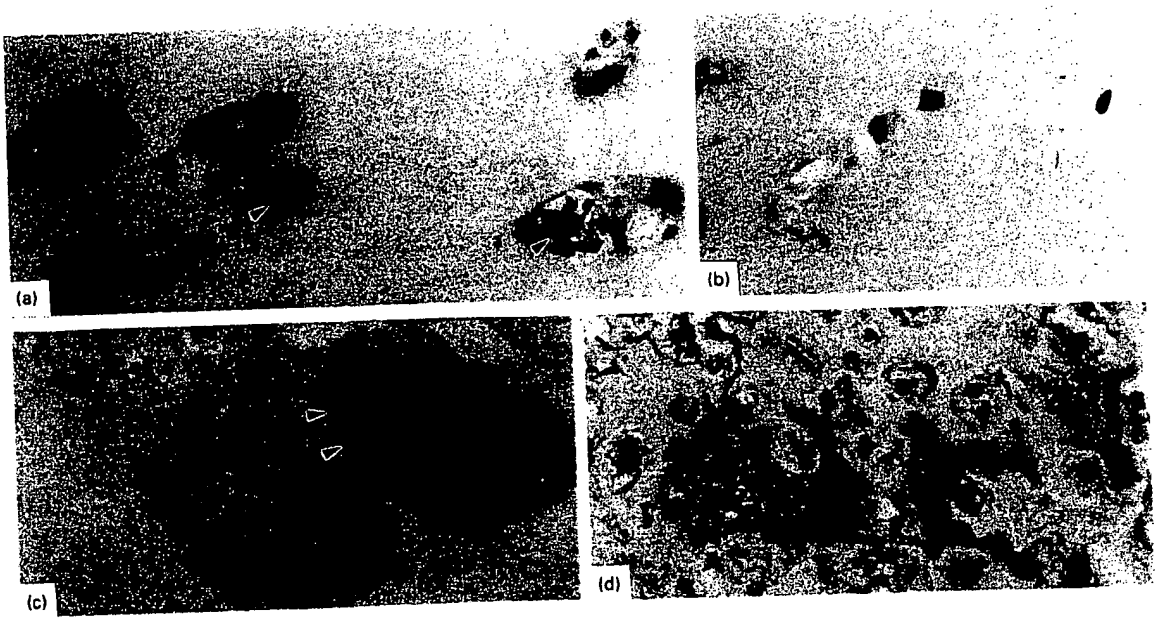


Figure 8 Immunolocalization of perlecan in human cartilage and chondrosarcoma tissues using a monoclonal antibody directed against domain III of human perlecan protein core

Notice the presence of immunoreactive perlecan in close association with normal human articular cartilage chondrocytes (**a**, arrowheads), while the entire cartilage matrix is unreactive. Panel (**b**) is a control section lacking the monoclonal antibody. Panels (**c**) and (**d**) represent two separate cases of human chondrosarcoma tissue showing intense perlecan staining around atypical chondrocytes (**c**, arrowheads) or as large deposits within the tumour (**d**, asterisk). (Original magnification: **a** and **b**, × 650; **c** and **d**, × 450).

glycans such as perlecan bind bFGF through the same oligosaccharide, the fact that the bFGF-specific oligosaccharide was isolated from the heparan sulphate released into the medium by human skin fibroblasts strongly implicates perlecan as a major source of this material.

In summary, the current picture of perlecan–growth factor interactions is quite complex and in a state of constant flux. As we gain more information about protein and carbohydrate isoforms and specific functional subsequences, we will be able to define more precisely the roles these interactions may play during organogenesis and development. The central theme of this research line is that the multiple autocrine or paracrine effector molecules might be influenced by the selective temporal and spatial expression of pericellular heparan sulphate proteoglycans during morphogenesis and development. A more subtle pathway would involve processing via mild degradation with proteases or endoglycosidases, thereby releasing the factors in a heparan sulphate-bound, active form.

NEW CLUES ABOUT FUNCTION: PERLECAN ISOFORMS

Alternatively-spliced variants in the mouse and nematode species

The perlecan story is complicated by the existence of variant forms in the mouse [1] and *Caenorhabditis elegans* [21] species. Comparison between domain IV of the mouse and human molecules shows that the mouse species lacks seven Ig repeats, from the middle of the fifth to the middle of the twelfth repeat (Figure 1). This generates a perlecan protein core of smaller size (approx. 396 versus approx. 467 kDa). Recently, however, three additional Ig-like repeats, aligning quite well with the human repeats, have been found in the mouse species and larger mRNA transcripts have been identified [60]. Because chromosomal *in situ* hybridization and somatic cell hybridization studies have all supported the existence of only a single perlecan gene, it is likely that alternate exon usage is the principal mechanism responsible for the generation of these transcripts. An extensive search by our laboratory for smaller perlecan variants in humans using RT-PCR and primers encompassing critical regions of domains I, II and IV, has so far failed to reveal any such species. By Northern blotting, we have constantly detected a single transcript of approx. 14 kb (cf. Figure 6). We can cautiously conclude from these data that the larger form of perlecan is the predominant species, and if smaller variants exist in human, they probably represent minor components or tissue-specific isoforms. This is in agreement with the fact that the complete human perlecan cDNA sequence is based on clones isolated from seven different cDNA libraries [19,20,24,25]. In addition, numerous RNAs and cDNA libraries have been screened in our laboratory and the amplified bands were all of the predicted size and sequence. It would be very interesting to determine whether a specific perlecan isoform might be linked to the transformed phenotype. Nevertheless, the high degree of conservation of the various perlecan domains between the human and the mouse species indicates that these modular units are important for perlecan function. This concept is further supported by the discovery of the *unc-52*, a miniaturized form of perlecan gene, in the nematode *C. elegans* [21]. This protein harbours three consecutive domains that resemble the LDL receptor, the laminin short chain and the N-CAM respectively, but is devoid of both the N- and C-terminal domains of perlecan (Figure 1). Domain I in the nematode, a stretch of 28 amino acids including a signal peptide, has little similarity to its mammalian counterpart, with the exception that both are hydrophilic and do not contain cysteine residues or internal repetitive sequences. In addition, the *C. elegans* domain I lacks the triplet SGD and thus it is not known whether this

species is synthesized as a proteoglycan or as a protein. Domains II and III are similar to the mammalian species with the exception of one less globular repeat in each domain. As in the mouse species, domain IV of *C. elegans* contains 14 Ig-like repeats with two additional repeats flanking domain II. The presence of a signal peptide and the immunological localization of *unc-52* protein in the nematode basement membrane strongly indicate that this gene product is an extracellular macromolecule. The nematode perlecan-like protein undergoes extensive alternative splicing with several isoforms varying in their C-termini [21]. The longest transcript consists of 26 exons that span approx. 15 kb of genomic DNA. The additional two smaller transcripts are generated by differential utilization of polyadenylation signals located approx. 8.5 kb apart and by differential splicing of the exons at the 3′ end of the *unc-52* gene [21]. All the exons encoding domain IV are located between the two polyadenylation signals, and thus the two larger transcripts encode proteins containing all or part of domain IV, whereas the smaller transcript encodes a truncated polypeptide that is completely devoid of domain IV repeats.

Implication of perlecan-like molecule in muscle cell biology

An unsuspected role of perlecan-like molecules derives from research on a commonly vilified organism, the worm, and opens the possibility that genetic mutations at the perlecan locus may contribute to skeletal muscle pathology in humans. Interestingly, mutations of the *unc-52* gene induce disruption of the skeletal muscle architecture of *C. elegans* [21]. Moerman and co-workers [21,117] have used suppression analysis to identify a new gene involved in maintaining the attachment of myofilaments to the cell surface and basement membrane of the body-wall muscle cells. Based on their complex pattern of complementation, two major types of mutations occur in this animal: a lethal form associated with a complete loss of function for at least one of the *unc-52* alleles and a viable, but paralysed, variant caused by the insertion of a Tc1 transposon into the eighth Ig-like repeat of domain IV [21]. The disorganized body-wall muscle phenotypes in both mutant forms suggest that perlecan-like molecules are involved in maintaining the structural integrity of the skeletal muscle. The findings in *C. elegans* indicate that the abnormal but viable phenotype is caused by the synthesis of a truncated *unc-52* protein, while the lethal phenotype is caused by the complete absence of the protein. At least in the nematode, the integrity of the N-CAM region is essential for full attachment of myofilaments to the basement membrane. Because of the widespread expression of mammalian perlecan in skeletal and smooth muscle, it is possible that some forms of human congenital myopathy may also be due to abnormally truncated forms of perlecan. Is there any genetic disease, pertinent to the muscular dystrophy *sensu lato*, that is linked to abnormal perlecan? This is an important question that may be addressed in the future by targeted disruption of the gene in transgenic animals.

PERLECAN IN HUMAN PATHOLOGY

Given the current concept that basement membranes provide directional anchors for cells and a scaffolding that fosters tissue morphogenesis, and given the recent realization that they originate through a process of cell assembly driven by 'mass-action' [68], it is not surprising that the deregulated expression of even a single vital constituent could lead to serious complications, and ultimately to a pathological phenotype. Below, we discuss contemporary knowledge regarding two examples of human pathological processes in which perlecan may play an active role.

Diabetic microangiopathy

In long-term diabetes mellitus a number of complications are inflicted on the diabetic individual. Among the most clinically significant are the microangiopathies. These are disruption of the normal function of vascular capillary beds. A hallmark of these disease processes is a significant thickening, or sometimes duplication, of the microvascular basement membrane (Figure 9), with chronic progression as a function of time. Paradoxically, this thickening of the basement membrane is accompanied by a loss of function, allowing charged serum molecules which are normally retained within the circulation to pass across the matrix. This produces systemic effects in skin and muscle, but the most marked clinical consequences are observed in the renal glomerulus in diabetic nephropathy and in the retina with hard exudate formation, microaneurysms, haemorrhaging and neovascularization and blindness in the proliferative end stage of diabetic retinopathy [118]. The physicochemical changes in the properties of the basement membrane were assumed to be linked to some change in matrix composition, and with much of the charge barrier present attributable to the heparan sulphate chains of proteoglycans [86], these macromolecules have become the subject of considerable interest in the aetiology of microangiopathies. A number of approaches have been developed in the investigation of these complications, many involving experimental models of diabetes which are either induced by chemical destruction of pancreatic islet cells or by the use of strains of animals bred to be genetically susceptible to the disease. Problems facing investigators were ones familiar to matrix researchers: the affected structures in most vascular beds were too diffusely distributed and too insoluble to yield adequate material for biochemical analysis. Most studies therefore focused on the kidney as the organ of choice; the glomerulus providing the most concentrated and abundant amounts of relatively pure capillary basement membrane. Incubation of isolated glomeruli from streptozotocin-induced diabetic and non-diabetic rats with radiolabelled sulphate has shown that the former glomeruli incorporate less sulphate into the basement membrane, while the total uronic acid content is unchanged [119,120], thereby indicating possible undersulphation of the glycosaminoglycan chains. As an indication that these effects might be somewhat more widespread, it has also been shown that liver heparan

Figure 9 Electron micrograph of a small capillary from a patient with long-standing diabetes mellitus

Notice the duplication of the basement membrane (multiple arrowheads) surrounding the endothelial cells (EC). The lumen of the blood vessel is indicated by an asterisk.

sulphate proteoglycans from streptozotocin-induced diabetic rats were also undersulphated, possibly through inhibition of N-deacetylase, one of the enzymes required for N-sulphation of glycosaminoglycans [121]. However, Klein et al. [122] found no decrease in sulphate-labelled proteoglycans in glomeruli from streptozotocin-treated rats, and suggested that alterations in the interactions of heparan sulphate proteoglycans with other basement membrane components were responsible for the decrease in heparan sulphate proteoglycan content.

In an attempt to circumvent problems related to quantity of material, some workers have examined the synthesis of proteoglycans in EHS tumours grown in diabetic host animals. Interestingly, EHS tumours from streptozotocin-treated diabetic mice incubated with high glucose showed a marked increase in radiolabelled sulphate incorporation into proteoglycans [123]. In contrast, other studies showed that in EHS tumours grown in genetically (non-insulin-dependent) diabetic [124] or streptozotocin-treated diabetic mice [125], a reduction in sulphate incorporation correlated with a reduction in the level of heparan sulphate proteoglycan protein core. However, using steady-state mRNA levels to examine protein-core synthesis in a mouse model of non-insulin-dependent diabetes, no absolute changes in kidney cortex levels of perlecan mRNA were observed [126], although there was proportionally less when compared with the elevated levels of type-IV collagen in diabetic animals with demonstrated glomerular basement membrane thickening.

In the few studies performed with human material, a similarly confusing picture emerges. In an early study [127], the amount of heparan sulphate in whole kidney cortex and medulla was found to be increased in some diabetic patients. However, other investigators [128] found a significantly reduced heparan sulphate content in the glomerular basement membrane of diabetics, with no change in the degree of sulphation.

To summarize, there is evidence for major alterations in heparan sulphate proteoglycan in diabetic nephropathy. These abnormalities can take place via: (i) an increase in sulphate incorporation; (ii) a decrease in charge via a decrease in protein-core biosynthesis or enhanced degradation; (iii) a decrease in sulphation alone; (iv) a dilution effect brought on by unbalanced synthesis of other basement membrane constituents; and (v) a loss of heparan sulphate proteoglycan following perturbations in the forces anchoring this macromolecule in the basement membrane matrix.

Taken at face value, these results are confusing and conflicting. It can be difficult, even in the light of more recent knowledge, to re-interpret the data presented in these studies given the differences in experimental approach and measurements made in some studies, which were omitted from others. A key question is to what extent the numerous sulphate incorporation experiments could be interpreted as reflecting perlecan biosynthesis/turnover. While clearly not the only proteoglycan present in all basement membranes, current evidence suggests that perlecan is the major heparan sulphate proteoglycan of the matrices affected by diabetic microangiopathy, including the glomerular basement membrane, and thus it is probably safe to assume that alterations to the perlecan molecule could contribute to the consequent pathology.

Tumour progression and invasion: perlecan in the metastatic cascade

It is becoming apparent that heparan sulphate proteoglycans are key players in many interactions between the cells and their environment. During tumour cell dissemination, these relationships become modulated, further enhancing the disorganization of the basement membrane. Given its polyhedric nature, perlecan holds a key position in the basement membrane to influence the processes leading to tumour invasion. Previous work on basement membrane heparan sulphate proteoglycans indicated that sulphated glycosaminoglycan chains are degraded at a faster rate by melanoma cells with high metastatic potential [129] by an endoglucuronidase or heparanase [130], reinforcing the functional importance of heparan sulphate chains in invasion. Recently, differences in the sulphation pattern of basement membrane proteoglycans were identified in Lewis lung carcinoma cells with high metastatic potential [131]. Evidence of a major role for heparan sulphate chains in tumour formation is further provided by the work of Esko and co-workers [132]. These authors generated mutant cell lines deficient in heparan sulphate chains and demonstrated that none of the mutants grew abnormally *in vitro*, thus suggesting that these glycosaminoglycans are not essential for growth. However, surprising results were obtained when the deficient cells were challenged to grow as tumours in athymic *nu/nu* mice. Whereas mutants grossly deficient in heparan sulphate synthesis were not tumorigenic, mutants with partially reduced levels were tumorigenic and required approx. 10% of the wild-type levels of heparan sulphate biosynthesis to express the malignant phenotype [132]. In general, any significant change in the sulphation pattern or enzymic degradation of the heparan sulphate may result in defects in assembly and stability of the basement membrane, especially in the surrounding endothelial cells, and may therefore facilitate the invasion/extravasation of tumour cells. There is no doubt that any alteration in this equilibrium will have an influence on the process of local invasion.

The protein core of perlecan is also capable of interacting with components of the matrix to affect cell adhesive behaviour. An emerging picture is that perlecan is directly involved in supporting the growth and invasion of tumour cells *in vivo* via its ability to capture and store angiogenic factors, such as bFGF [104]. Studies of cell-surface proteoglycans on human melanomas have shown that a predominance of heparan sulphate proteoglycan is a marker for a more aggressive phenotype [133]. Also, perlecan isolated from the EHS tumour stimulates invasiveness of melanoma cells in an *in vitro* assay [134]. The growth of transformed endothelial cells, but not of normal cells, on reconstituted basement membranes is severely suppressed when perlecan is degraded [135]. Furthermore, we and others have observed abundant deposits of perlecan in the stroma of a number of malignant tissues including breast and colon cancer (cf. Figures 7d and 7e respectively). Pronounced stromal reaction was observed in human colon carcinoma with perlecan and type-IV collagen forming fibrillar deposits, a reaction not observed in colonic polyps [136]. In a series of experiments using the prostate carcinoma cell line PC-3 to induce tumour xenografts in immunodeficient severe combined immunodeficient ('SCID') mice, we found that the newly formed microvessels in the tumour stroma were intensely reactive with a monoclonal antibody against perlecan (Figures 7f and 7g). Because our monoclonal antibody does not recognize mouse perlecan, as shown by the lack of staining of the mouse skin basement membrane (Figure 7f, asterisks), we conclude that the perlecan that is deposited around the tumour microvessels derives from the human PC-3 carcinoma cells. In contrast, the endothelial cells lining the tumour microvessels were of mouse origin as they failed to react with a monoclonal antibody against human factor VIII (results not shown). In summary, these data suggest that the prostate carcinoma cells synthesize perlecan, as also proven by the RT-PCR data (cf. Figure 6), and that this molecule induces or favours neovascularization of the transplanted tumour. A gra-

dient of immunoreactivity was also visible, with the most reactive microvessels localized at or around the growing edge of the tumour xenograft (Figure 7f). If this occurs in human tumours, then neoplastic clones with an ability to over-express perlecan would have a growth advantage over clones that lack this phenotypic trait. The sequestration of bFGF by perlecan and its release by tumour-cell-derived enzymes [103,104] could constitute a plausible autocrine or paracrine loop influencing local tumour growth or invasion. Accordingly, bFGF has been localized to various human tumours in the digestive [137] and nervous [138] systems. The abnormal deposition of bFGF in the extracellular matrix of gastric cancers was related primarily to areas of increased vascular permeability and active angiogenesis [137]. The close association between tumour cells and the vascular basement membrane observed in gastric cancer with liver metastases has been proposed to be specific for a more aggressive behaviour [139], whereas lung carcinoma cells with higher metastatic propensity adhere to a subendothelial matrix more efficiently than cells with lower metastatic potential [140]. These studies are in agreement with our observation in tumour xenografts discussed above and suggest that the ability of tumour cells to form a well-defined basement membrane is positively correlated with their adhesiveness to the vascular basement membrane *in vivo*.

CONCLUDING REMARKS

The recent advances in the chemistry and biology of heparan sulphate proteoglycans and their ligand interactions are having a considerable scientific and clinical impact. Contemporary evidence indicates that (i) perlecan is a highly conserved gene product with isoforms present in the nematode and mouse, (ii) that this molecule can exist as a glycoprotein and (iii) that its expression is not restricted to basement membranes. The composite, multidomain perlecan is made up of a montage of protein modules that are evolutionarily related to molecules involved in nutrient metabolism, mitogenesis and cell adhesion. The emerging portrait of perlecan biology is based principally on perlecan's ability to interact with cells and their products. This active participation occurs via permissive structural hierarchies in which the level of interaction, whether homotypic or heterotypic, is dictated by the structural motifs and their constitutive binding characteristics. Characterization of the genomic structure, chromosomal mapping of the gene, discovery of several RFLPs and of alternatively spliced variants are important conceptual advances that will undoubtedly facilitate a more comprehensive understanding of this molecule. The stage is now set to unravel the role of perlecan in fundamental biological processes wherein tissue remodelling occurs, inasmuch as this chimeric molecule is utilized by cells during embryonic development, wound repair and cancer growth. Systematic studies are needed to learn about the function of the individual protein modules and to develop transgenic animals with deregulated or abrogated perlecan expression. Collectively these studies should provide novel avenues of research and perhaps discover unforeseeable functions for this important molecule. One of the future challenges is to define the signal transducing pathways through which perlecan can activate specific sets of genes in different cell types.

The original research was supported by grants RO1 CA-39481 and RO1 CA-47282 from the National Institutes of Health and by a Faculty Research Award (FRA-376) from the American Cancer Society (R.V.I.). We are deeply indebted to Dr. C. C. Clark for critical reading of the manuscript. We would like to express our appreciation to our colleagues J. Hassell, R. Tuan, R. Schwarting, W. O. McBride, E. Lattime, and M.-L. Chu for their contributions to the work on perlecan.

REFERENCES

1 Noonan, D. M., Fulle, A., Valente, P., Cai, S., Horigan, E., Sasaki, M., Yamada, Y. and Hassell, J. R. (1991) J. Biol. Chem. **266**, 22939–22947
2 Bernfield, M. R., Banerjee, S. D. and Cohn, R. (1972) J. Cell Biol. **52**, 674–689
3 Gordon, J. R. and Bernfield, M. R. (1980) Dev. Biol. **74**, 118–135
4 Kanwar, Y. S. and Farquhar, M. G. (1979) Proc. Natl. Acad. Sci. U.S.A. **76**, 1303–1307
5 Hassell, J. R., Robey, P. G., Barrach, H. J., Wilczek, J., Rennard, S. I. and Martin, G. R. (1980) Proc. Natl. Acad. Sci. U.S.A. **77**, 4494–4498
6 Kleinman, H. K., McGarvey, M. L., Liotta, L. A., Robey, P. G., Tryggvason, K. and Martin, G. R. (1982) Biochemistry **24**, 6188–6193
7 Kleinman, H. K., McGarvey, M. L., Hassell, J. R., Star, V. L., Cannon, F. B., Laurie, G. W. and Martin, G. R. (1986) Biochemistry **25**, 312–318
8 Paulsson, M., Yurchenco, P. D., Ruben, G. C., Engel, J. and Timpl, R. (1987) J. Mol. Biol. **197**, 297–313
9 Iozzo, R. V. and Hassell, J. R. (1989) Arch. Biochem. Biophys. **269**, 239–249
10 Noonan, D. M., Horigan, E. A., Ledbetter, S. R., Vogeli, G., Sasaki, M., Yamada, Y. and Hassell, J. R. (1988) J. Biol. Chem. **263**, 16379–16387
11 Iozzo, R. V. (1984) J. Cell Biol. **99**, 403–417
12 Heremans, A., Cassiman, J.-J., Van Den Berghe, H. and David, G. (1988) J. Biol. Chem. **263**, 4731–4739
13 Saku, T. and Furthmayr, H. (1989) J. Biol. Chem. **264**, 3514–3523
14 Jalkanen, M., Rapraeger, A. and Bernfield, M. (1988) J. Cell Biol. **106**, 953–962
15 Kato, M., Kolke, Y., Suzuki, S. and Kimata, K. (1988) J. Cell Biol. **106**, 2203–2210
16 Klein, D. J., Brown, D. M., Oegema, T. R., Brenchley, P. E., Anderson, J. C., Dickinson, M. A. J., Horigan, E. A. and Hassell, J. R. (1988) J. Cell Biol. **106**, 963–970
17 Ledbetter, S. R., Tyree, B., Hassell, J. R. and Horigan, E. A. (1985) J. Biol. Chem. **260**, 8106–8113
18 Gilbert, W. (1985) Science **228**, 823–824
19 Murdoch, A. D., Dodge, G. R., Cohen, I., Tuan, R. S. and Iozzo, R. V. (1992) J. Biol. Chem. **267**, 8544–8557
20 Kallunki, P. and Tryggvason, K. (1992) J. Cell Biol. **116**, 559–571
21 Rogalski, T. M., Williams, B. D., Mullen, G. P. and Moerman, D. G. (1993) Genes Dev. **7**, 1471–1484
22 Cohen, I., Grässel, S., Murdoch, A. D. and Iozzo, R. V. (1993) Proc. Natl. Acad. Sci. U.S.A. **90**, 10404–10408
23 Wintle, R. F., Kisilevsky, R., Noonan, D. and Duncan, A. M. V. (1990) Cytogenet. Cell Genet. **54**, 60–61
24 Dodge, G. R., Kovalszky, I., Chu, M.-L., Hassell, J. R., McBride, O. W., Yi, H. F. and Iozzo, R. V. (1991) Genomics **10**, 673–680
25 Kallunki, P., Eddy, R. L., Byers, M. G., Kestilä, M., Shows, T. B. and Tryggvason, K. (1991) Genomics **11**, 389–396
26 Chakravarti, S., Phillips, S. L. and Hassell, J. R. (1991) Mammalian Genome **1**, 270–272
27 Bruns, G. A. P. and Sherman, S. L. (1989) Cytogenet. Cell Genet. **51**, 67–90
28 Greene, M. H., Goldin, L. R., Clark, W. H., Jr., Lovrien, E., Kraemer, K. H., Tucker, M. A., Elder, D. E., Fraser, M. C. and Rowe, S. (1983) Proc. Natl. Acad. Sci. U.S.A. **80**, 6071–6075
29 Dracopoli, N. C., Harnett, P., Bale, S. J., Stanger, B. Z., Tucker, M. A., Housman, D. E. and Kefford, R. F. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 4614–4618
30 Fong, C.-T., Dracopoli, N. C., White, P. S., Merrill, P. T., Griffith, R. C., Housman, D. E. and Brodeur, G. M. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 3753–3757
31 Weith, A., Martinsson, T., Cziepluch, C., Brüderlein, S., Amler, L. C. and Berthold, F. (1989) Genes Chromosomes Cancer **1**, 159–166
32 Mathew, C. G. P., Smith, B. A., Thorpe, K., Wong, Z., Royle, N. J., Jeffreys, A. J. and Ponder, B. A. J. (1987) Nature (London) **328**, 524–526
33 Genuardi, M., Tsihira, H., Anderson, D. E. and Saunders, G. F. (1989) Am. J. Hum. Genet. **45**, 73–82
34 Leister, I., Weith, A., Brüderlein, S., Cziepluch, C., Kangwanpong, D., Schlag, P. and Schwab, M. (1990) Cancer Res. **50**, 7232–7235
35 Bird, A. P. (1986) Nature (London) **321**, 209–213
36 Mitchell, P. J. and Tjian, R. (1989) Science **245**, 371–378
37 Vuolteenado, R., Chow, L. T. and Tryggvason, K. (1990) J. Biol. Chem. **265**, 15611–15616
38 Kallunki, T., Ikonen, J., Chow, L. T., Kallunki, P. and Tryggvason, K. (1991) J. Biol. Chem. **266**, 221–228
39 Colwell, G., Li, B., Forrest, D. and Brackenbury, R. (1992) Genomics **14**, 875–882
40 Gallagher, J. T. (1989) Curr. Opin. Cell Biol. **1**, 1201–1218
41 Ruoslahti, E. and Yamaguchi, Y. (1991) Cell **64**, 867–869
42 Bernfield, M., Kokenyesi, R., Kato, M., Hinkes, M. T., Spring, J., Gallo, R. L. and Lose, E. J. (1992) Annu. Rev. Cell Biol. **8**, 365–393
43 Mali, M., Jaakkola, P., Arvilommi, A.-M. and Jalkanen, M. (1990) J. Biol. Chem. **265**, 6884–6889

44 Carey, D. J., Evans, D. M., Stahl, R. C., Asundi, V. K., Conner, K. J., Garbes, P. and Cizmeci-Smith, G. (1992) J. Cell Biol. **117**, 191–201
45 David, G., Lories, V., Decock, B., Marynen, P., Cassiman, J. J. and Van Den Berghe, H. (1990) J. Cell Biol. **111**, 3165–3176
46 Hassell, J. R., SundarRaj, N. and Ledbetter, S. (1993) Mol. Biol. Cell **4**, 349a
47 Südhof, T. C., Goldstein, J. L., Brown, M. S. and Russell, D. W. (1985) Science **228**, 815–822
48 Stanley, K. K. (1988) Curr. Topics Microbiol. Immunol. **140**, 49–65
49 Raychowdhury, R., Niles, J. L., McCluskey, R. T. and Smith, J. A. (1989) Science **244**, 1163–1165
50 Strickland, D. K., Ashcom, J. D., Williams, S., Burgess, W. H., Migliorini, M. and Argraves, W. S. (1990) J. Biol. Chem. **265**, 17401–17404
51 Yochem, J. and Greenwald, I. (1993) Proc. Natl. Acad. Sci. U.S.A. **90**, 4572–4576
52 Bates, P., Young, J. A. T. and Varmus, H. E. (1993) Cell **74**, 1043–1051
53 Wight, T. N., Kinsella, M. G. and Qwarnström, E. A. (1992) Curr. Opin. Cell Biol. **4**, 793–801
54 Williams, K. J., Fless, G. M., Petrie, K. A., Snyder, M. L., Brocia, R. W. and Swenson, T. L. (1992) J. Biol. Chem. **267**, 13284–13292
55 Olgemöller, B., Schleicher, E. D., Schwaabe, S., Guretzki, H.-J. and Gerbitz, K. D. (1990) FEBS Lett. **264**, 37–39
56 Iozzo, R. V. (1988) J. Cell. Biochem. **37**, 61–78
57 Sasaki, M., Kleinman, H. K., Huber, H., Deutzmann, R. and Yamada, Y. (1988) J. Biol. Chem. **263**, 16536–16544
58 Haaparanta, T., Uitto, J., Ruoslahti, E. and Engvall, E. (1991) Matrix **11**, 151–160
59 Cunningham, B. A., Hemperly, J. J., Murray, B. A., Prediger, E. A., Brackenbury, R. and Edelman, G. M. (1987) Science **236**, 799–806
60 Noonan, D. M. and Hassell, J. R. (1993) Kidney Int. **43**, 53–60
61 Yurchenco, P. D., Cheng, Y.-S. and Ruben, G. C. (1987) J. Biol. Chem. **262**, 17668–17676
62 Rutishauser, U., Acheson, A., Hall, A. K., Mann, D. M. and Sunshine, J. (1988) Science **240**, 53–57
63 Traut, T. W. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 2944–2948
64 Appella, E., Weber, I. T. and Blasi, F. (1988) FEBS Lett. **231**, 1–4
65 Ushkaryov, Y. A., Petrenko, A. G., Geppert, M. and Südhof, T. C. (1992) Science **257**, 50–56
66 Ushkaryov, Y. A. and Südhof, T. C. (1993) Proc. Natl. Acad. Sci. U.S.A. **90**, 6410–6414
67 Rupp, F., Payan, D. G., Magill-Solc, C., Cowan, D. M. and Scheller, R. H. (1991) Neuron, **6**, 811–823
68 Yurchenco, P. D. and Schittny, J. (1990) FASEB J. **4**, 1577–1590
69 Panayotou, G., End, P., Aumailley, M., Timpl, R. and Engel, J. (1989) Cell **56**, 93–101
70 Skubitz, A. P. N., Letourneau, P. C., Wayner, E. and Furcht, L. T. (1991) J. Cell Biol. **115**, 1137–1148
71 Danielson, K. G., Martinez-Hernandez, A., Hassell, J. R. and Iozzo, R. V. (1992) Matrix **11**, 22–35
72 Hassell, J. R., Schrecengost, P. K., Rada, J. A., SundarRaj, N., Sossi, G. and Thoft, R. A. (1992) Invest. Opthalmol. Vis. Sci. **33**, 547–557
73 Isemura, M., Sato, N., Yamaguchi, Y., Aikawa, J., Munakata, H., Hayashi, N., Yosizawa, Z., Nakamura, T., Kubota, A., Arakawa, M. and Hsu, C.-C. (1987) J. Biol. Chem. **262**, 8926–8933
74 Hunter, D. D., Porter, B. E., Bulock, J. W., Adams, S. P., Merlie, J. P. and Sanes, J. R. (1989) Cell **59**, 905–913
75 Dziadek, M., Fujiwara, S., Paulsson, M. and Timpl, R. (1985) EMBO J. **4**, 905–912
76 Carson, D. D., Tang, J.-P. and Julian, J. (1993) Dev. Biol. **155**, 97–106
77 Murdoch, A. D., Liu, B., Schwarting, R., Tuan, R. S. and Iozzo, R. V. (1994) J. Histochem. Cytochem. **42**, 239–249
78 Couchman, J. R. (1987) J. Cell Biol. **105**, 1901–1916
79 Couchman, J. R. and Ljubimov, A. V. (1989) Matrix **9**, 311–321
80 Horiguchi, Y., Fine, J.-D. and Couchman, J. R. (1991) Br. J. Dermatol. **124**, 410–414
81 Rescan, P.-Y., Loréal, O., Hassell, J. R., Yamada, Y., Guillouzo, A. and Clément, B. (1993) Am. J. Pathol. **142**, 199–208
82 Laurie, G. W., Leblond, C. P., Inoue, S., Martin, G. R. and Chung, A. (1984) Am. J. Anat. **169**, 463–481
83 Hagen, S. G., Michael, A. F. and Butkowski, R. J. (1993) J. Biol. Chem. **268**, 7261–7269
84 Simon-Assmann, P., Bouziges, F., Vigny, M. and Kedinger, M. (1989) J. Cell Biol. **109**, 1837–1848
85 Stow, J. L., Sawada, H. and Farquhar, M. G. (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 3296–3300
86 Kanwar, Y. S., Linker, A. and Farquhar, M. G. (1980) J. Cell Biol. **86**, 688–693
87 Grant, D. S. and Leblond, C. P. (1988) J. Histochem. Cytochem. **36**, 271–283
88 Heremans, A., van der Schueren, B., de-Cock, B., Paulsson, M., Cassiman, J. J., van den Berghe, H. and David, G. (1989) J. Cell Biol. **109**, 3199–3211
89 Inoue, S., Grant, D. and Leblond, C. P. (1989) J. Histochem. Cytochem. **37**, 597–602
90 Grant, D. S., Leblond, C. P., Kleinman, H. K., Inoue, S. and Hassell, J. R. (1989) J. Cell Biol. **108**, 1567–1574
91 Iozzo, R. V., Kovalszky, I., Hacobian, N., Schick, P. K., Ellingson, J. S. and Dodge, G. R. (1990) J. Biol. Chem. **265**, 19980–19989
92 Battaglia, C., Aumailley, M., Mann, K., Mayer, U. and Timpl, R. (1993) Eur. J. Cell Biol. **61**, 92–99
93 Battaglia, C., Mayer, U., Aumailley, M. and Timpl, R. (1992) Eur. J. Biochem. **208**, 359–366
94 Reinhardt, D., Mann, K., Nischt, R., Fox, J. W., Chu, M.-L., Krieg, T. and Timpl, R. (1993) J. Biol. Chem. **268**, 10881–10887
95 Vlodavsky, I., Bar-Shavit, R., Ishai-Michaeli, R., Bashkin, P. and Fuks, Z. (1991) Trends Biochem. Sci. **16**, 268–271
96 Hassell, J. R., Leyshon, W. C., Ledbetter, S. R., Tyree, B., Suzuki, S., Kato, M., Kimata, K. and Kleinman, H. K. (1985) J. Biol. Chem. **260**, 8098–8105
97 Timpl, R. (1993) Experientia **49**, 417–428
98 Heremans, A., de-Cock, B., Cassiman, J. J., van den Berghe, H. and David, G. (1990) J. Biol. Chem. **265**, 8716–8724
99 Clément, B., Segui-Real, B., Hassell, J. R., Martin, G. R. and Yamada, Y. (1989) J. Biol. Chem. **264**, 12467–12471
100 Hayashi, K., Madri, J. A. and Yurchenco, P. D. (1992) J. Cell Biol. **119**, 945–959
101 Turnbull, J. E. and Gallagher, J. T. (1993) Biochem. Soc. Trans. **21**, 477–482
102 Esko, J. D. (1991) Curr. Opin. Cell Biol. **3**, 805–816
103 Folkman, J., Klagsbrun, M., Sasse, J., Wadzinski, M., Ingber, D. and Vlodavsky, I. (1988) Am. J. Pathol. **130**, 393–400
104 Vigny, M., Olier-Hartmann, M. P., Lavigne, M., Fayein, N., Jeanny, J. C., Laurent, M. and Courtois, Y. (1988) J. Cell. Physiol. **137**, 321–328
105 Bashkin, P., Doctrow, S., Klagsbrun, M., Svahn, C. M., Folkman, J. and Vlodavsky, I. (1989) Biochemistry **28**, 1737–1743
106 Saksela, O., Moscatelli, D., Sommer, A. and Rifkin, D. B. (1988) J. Cell Biol. **107**, 743–751
107 Saksela, O. and Rifkin, D. B. (1990) J. Cell Biol. **110**, 767–775
108 Folkman, J. and Shing, Y. (1992) J. Biol. Chem. **267**, 10931–10934
109 Sasisekharan, R., Moses, M. M., Nugent, M. A., Cooney, C. L. and Langer, R. (1994) Proc. Natl. Acad. Sci. U.S.A. **91**, 1524–1528
110 Moscatelli, D. (1988) J. Cell Biol. **107**, 753–759
111 Lortat-Jacob, H., Kleinman, H. K. and Grimaud, J.-A. (1991) J. Clin. Invest. **87**, 878–883
112 Austria, M. R. and Couchman, J. R. (1993) Mol. Biol. Cell **4**, 184a
113 Dodge, G. R., Kovalszky, I., Hassell, J. R. and Iozzo, R. V. (1990) J. Biol. Chem. **265**, 18023–18029
114 Yayon, A., Klagsbrun, M., Esko, J. D., Leder, P. and Ornitz, D. M. (1991) Cell **64**, 841–848
115 Ornitz, D. M., Yayon, A., Flanagan, J. G., Svahn, C. M., Levi, E. and Leder, P. (1992) Mol. Cell. Biol. **12**, 240–247
116 Turnbull, J. E., Fernig, D. G., Ke, Y., Wilkinson, M. C. and Gallagher, J. T. (1992) J. Biol. Chem. **267**, 10337–10341
117 Gilchrist, E. J. and Moerman, D. G. (1992) Genetics **132**, 431–442
118 Bloodworth, J. M. B. (1962) Diabetes **11**, 1–22
119 Cohen, M. P. and Surma, M. L. (1981) J. Lab. Clin. Med. **98**, 715–722
120 Kanwar, Y. S., Rosenzweig, L. J., Linker, A. and Jakubowski, M. L. (1983) Proc. Natl. Acad. Sci. U.S.A. **80**, 2272–2275
121 Kjellén, L., Bielefeld, D. and Höök, M. (1983) Diabetes **32**, 337–342
122 Klein, D. J., Oegema, T. R., Jr. and Brown, D. M. (1989) Diabetes **38**, 130–139
123 Pihlajaniemi, T., Myllylä, R., Kivirikko, K. I. and Tryggvason, K. (1982) J. Biol. Chem. **257**, 14914–14920
124 Rohrbach, D. H., Hassell, J. R., Kleinman, H. K. and Martin, G. R. (1982) Diabetes **31**, 185–188
125 Rohrbach, D. H., Wagner, C. W., Star, V. L., Martin, G. R., Brown, K. S. and Yoon, J.-W. (1983) J. Biol. Chem. **258**, 11672–11677
126 Ledbetter, S., Copeland, E. J., Noonan, D., Vogeli, G. and Hassell, J. R. (1990) Diabetes **39**, 196–203
127 Berenson, G. S., Ruiz, H., Dalferes, E. R., Jr., Dugan, F. A. and Radhakrishnamurthy, B. (1970) Diabetes **19**, 161–170
128 Parthasarathy, N. and Spiro, R. G. (1982) Diabetes **31**, 738–741
129 Nakajima, M., Irimura, T., Di Ferrante, D., Di Ferrante, N. and Nicolson, G. L. (1983) Science **220**, 611–612
130 Nakajima, M., Irimura, T., Di Ferrante, D. and Nicolson, G. L. (1984) J. Biol. Chem. **259**, 2283–2290
131 Nakanishi, H., Oguri, K., Yoshida, K., Itano, N., Takenaga, K., Kazama, T., Yoshida, A. and Okayama, M. (1992) Biochem. J. **288**, 215–224
132 Esko, J. D., Rostand, K. S. and Weinke, J. L. (1988) Science **241**, 1092–1096

133 Timar, J., Ladanyi, A., Lapis, K. and Moczar, M. (1992) Am. J. Pathol. **141**, 467–474
134 Marchetti, D., Menter, D., Jin, L., Nakajima, M. and Nicolson, G. L. (1993) Int. J. Cancer **55**, 692–699
135 Imamura, T., Tokita, Y. and Mitsui, Y. (1991) Cell Struct. Funct. **16**, 225–230
136 Ljubimov, A. V., Bartek, J., Couchman, J. R., Kapuller, L. L., Veselov, V. V., Kovarik, J., Perevoshchikov, A. G. and Krutovskikh, V. A. (1992) Int. J. Cancer **50**, 562–566
137 Ohtani, H., Nakamura, S., Watanabe, Y., Mizoi, T., Saku, T. and Nagura, H. (1993) Lab. Invest. **68**, 520–527
138 Paulus, W., Grothe, C., Sensenbrenner, M., Janet, T., Baur, I., Graf, M. and Roggendorf, W. (1990) Acta Neuropathol. **79**, 418–423
139 Nakanishi, H., Okayama, M., Oguri, K., Hayashi, K., Tateno, H. and Hosoda, S. (1991) Virchows Archiv. A. Pathol. Anat. **418**, 531–538
140 Nakanishi, H., Takenaga, K., Oguri, K., Yoshida, A. and Okayama, M. (1992) Virchows Archiv. A. Pathol. Anat. **420**, 163–170
141 Burgeson, R. E., Chiquet, M., Deutzmann, R., Ekblom, P., Engel, J., Kleinman, H. K., Martin, G. R., Ortonne, J. P., Paulsson, M., Sanes, J. R., Timpl, R., Tryggvason, K., Yamada, Y. and Yurchenco, P. D. (1994) Matrix Biol. **14**, 209–211
142 David, G., Van Der Schueren, B., Marynen, P., Cassiman, J. J. and Van Den Berghe, H. (1992) J. Cell Biol. **118**, 961–969
143 López-Casillas, F., Cheifetz, S., Doody, J., Andres, J. L., Lane, W. S. and Massagué, J. (1991) Cell **67**, 785–795